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PHYSIOLOGICAL ZOÖLOGY

Volume XXIV OCTOBER 1951 Number 4

THE CAUSES OF FIGHTING IN MICE AND RATS[1]

J. P. SCOTT AND EMIL FREDERICSON[2]

Division of Behavior Studies, Hamilton Station, Roscoe B. Jackson Memorial Laboratory

INTRODUCTION

All behavior may be considered as an attempt of an organism to adjust to changed conditions, whether internal or external. When fighting behavior is analyzed, it is found to be one of several patterns of behavior which may provide some measure of adjustment when two organisms come into conflict. Other common and closely related alternate patterns are escape behavior, defensive behavior, and passivity. It is difficult to consider any of these without the others, and it is with this general group of behavioral adjustments, which may be given the name "agonistic behavior," that this paper is concerned.

These patterns of behavior have probably been most extensively and thoroughly studied in two laboratory animals, *Rattus norvegicus* and *Mus musculus;* and on the basis of facts collected from these two rather closely related species it is possible to arrive at certain interesting generalizations.

It almost goes without saying that various species of animals have various special methods for adjusting to environmental changes. The fighting behavior of mice and rats has particular usefulness for science because it can be readily and easily studied. Mice especially can be readily induced to fight fiercely but, at the same time, do little damage to their opponents unless the fight is prolonged; and they can be readily controlled and separated.

An attempt to understand patterns of agonistic behavior in two closely related species should contribute much to a general understanding of these phenomena. Consideration of the factors affecting fighting and its ultimate causes in rats and mice immediately raises problems of the broadest general interest. For example, what is the real use of fighting? Darwin's hypothesis of advantageous individual competitive behavior must be re-examined in the light of modern knowledge of social organization. Why do males fight more than females, and are there other hereditary differences in aggressiveness? Is there an inborn and unsuppressible "drive" for fighting? How do training and heredity interact to modify fighting? Why do mice generally form dominance hierarchies with one dominant and several subordinate animals in-

[1] This study was supported in part by a research grant from the National Institute of Mental Health, U.S. Public Health Service.

[2] The authors wish to thank the following workers in the field for a critical reading of the manuscript: W. C. Allee, Benson Ginsburg, Elizabeth Beeman, J. P. Seward, John B. Calhoun, and Marvin Kahn.

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stead of straight-line hierarchies? How can fighting be controlled?

The following paper is an attempt to answer some of these questions by conducting a systematic investigation of available knowledge concerning the various factors which affect agonistic behavior in the two species, to organize these facts on a firm theoretical basis, and to indicate points where new research is needed.

DESCRIPTION OF PATTERNS OF AGONISTIC BEHAVIOR

The behavior patterns which may occur in conflict situations may be first described for mice. This applies to situations in which the conflict is between one mouse and another without involving a second species, and the behavior may be classified into three groups.

A. PRELIMINARY BEHAVIOR

Under certain situations actual fighting does not begin immediately but is preceded by the following events:

1. *Hair-fluffing.*—The hair literally stands on end over most of the body, with no emphasis on particular regions, as in the cat and dog. The amount of hair-fluffing is usually difficult to determine, and a poorly groomed animal may give somewhat the same appearance.

2. *Tail-rattling.*—The mouse rapidly twitches its tail from side to side, so that it takes on a wavy appearance. If the tail happens to strike against some hard object a loud rattling sound is produced. Both this and hair-fluffing appear to be involuntary behavior patterns, which other mice may learn to observe as warning signals, and occur in situations in which the mouse is hesitating between attack and escape reactions (Scott, 1947).

3. *Mincing.*—This type of behavior apparently occurs frequently when mice are fought in round-robin fashion (Beeman and Allee, 1945). Before attacking, the mouse circles around or near the opponent, with the body humped up and taking short, rapid steps. This behavior may be interpreted as a combination of attacking and defensive postures, or it may be a warning signal similar to the above. It is usually found in animals which have experienced both victory and defeat.

B. ATTACK

If two mice attack at once, they bite, scratch, and wrestle, often so rapidly that the eye cannot follow their movements. If one attacks and the other runs away, the first will chase after the second, biting and striking with the teeth when he catches up.

C. DEFENSIVE AND ESCAPE BEHAVIOR

When a mouse is beaten, it squeaks when attacked and, if the space is large enough, will run away. If shelter is provided, it may hide from the victorious mouse. In small barren cages where no such escape is possible, the mouse will rear up on the hind feet, holding out the front paws toward the aggressor and remaining motionless until attacked, when it may squeak and jump. This is a very characteristic and easily recognized pattern of behavior and has been called the "submission reaction" because it is a very reliable indicator of a dominance-subordination relationship (Ginsburg and Allee, 1942).[3] Scott (1946) interprets this

[3] Ginsburg and Allee described this attitude in the same way, except that one foreleg is said to be close to the body and the other extended stiffly. The authors of the present paper have never observed this to occur consistently. However, this is a very minor point, and the general facts appear to be that the attitude of defense exhibited by an animal in its first fight becomes more and more stereotyped with repeated defeats, until it assumes this posture in a mechanical way similar to a reflex action which may be called a "submission reaction."

behavior as an attitude of defense. If a beaten mouse is driven into a corner or small space, he may roll over on the back and hold all four feet in the air. This is simply an extension of the defensive attitude.

There are certain commonly observed patterns of behavior in mice which may be confused with fighting.

1. *Investigation.*—When strange mice meet, one animal may nose the other in various parts of the body.

2. *Grooming.*—Mice tend to clean one another's fur with the claws and teeth, and this may appear very similar to biting. In some cases rough grooming is apparently substituted for aggression.

3. *Sexual behavior.*—Sexual mounting and chasing may appear very similar to an attack, and in some cases this may start fights between males.

Essentially the same patterns of behavior can be seen in rats, except that tail-rattling has not been reported for these animals. The defense posture is very characteristic, but rats often rear up together, so that it is difficult to tell which is the dominant and which the subordinate animal. Another difference is that playful fighting is very common in young rats and has never been observed in young mice. Calhoun (1948) has reported that rats under wild conditions will administer "psychological drubbing" or mild attacks on the young animals. This also has not been reported for mice. Fighting in most of the tame strains of rats appears to be much less vigorous than in tame strains of mice.

The behavior patterns described appear to be largely unlearned reactions, as they may be seen fully developed in mice which are fighting for the first time. An experienced and successful fighter shows little change in behavior, except that he tends to become more vigorous and to bite more viciously and more effectively.

When any two animals are repeatedly placed in conflict situations, their behavior is soon organized into social dominance. This phenomenon may be defined as habitual control of the behavior of one animal by another by means of force, fighting, or threats. This control in the mouse usually includes the more or less complete inhibition of the patterns of attack, so that the subordinate mouse fights only when attacked or not at all and confines its behavior to defense and escape reactions. The same definition will apply to the behavior of rats.

In the scientific papers on the subject, considerable attention has been devoted to so-called "behavior traits." A "trait" may be defined as the consistent appearance of a certain pattern or group of patterns of behavior when an individual is put into a given situation. This consistency may be theoretically due to either heredity or habit formation or a combination of the two.

Most authors have used popular terminology, and this may at first be confusing to the reader in this field of scientific literature. Fortunately, the terms have been used fairly consistently. Where the situation is one of conflict between mice or between rats, the tendency to attack is called "aggressiveness," and the tendency to run away or escape is called "timidity." A lack of conflict behavior is called "peacefulness."

On the other hand, where the conflict lies between mice and people, or rats and people, the tendency of the nonhuman animal to attack has been called "savageness," and the tendency to escape has been called "wildness," while an absence of conflict behavior is called "tameness."

Several interesting problems come out of a consideration of the trait approach to agonistic behavior. One of these is the problem of measuring native aggressive tendencies, which obviously are modified

by social dominance as soon as the animal has had any experience in fighting. Native aggressiveness can be measured only at the time of the first fight, and even here may be modified by previous training (such as handling, in the case of mice). Another problem is that of how general such behavior traits may be. Is an animal which is very aggressive also very savage? The fact that the same animal may be dominant in one situation and subordinate in another indicates that behavior traits may have to be described only in terms of the special situations and social relationships which occur in a given animal's life. A third interesting problem is that of the connection between the traits which appear to be opposite but are actually alternative responses to the same situation. Is an animal which is more timid in one situation also more aggressive in another situation? Or are aggressiveness and timidity modified independently by various factors? These problems will be discussed under the section on heredity below.

METHODS AND TECHNIQUES

Hall has summarized the techniques for studying this problem in his 1941 paper. This evidence and that which is found in subsequent experimental work make it clear that two basic experimental methods have been used. One is to take animals which have been trained to fight and vary the different factors which are supposed to affect the amount and incidence of fighting. The other basic method is to take animals which are naïve and untrained and try out the effects of similar experimental factors. These two methods give very different results, since the first tends to test the importance of training and habit formation and the second tends to test for the presence of innate predisposing tendencies.

Among training methods two types have chiefly been used. One is the "round-robin" method, in which each mouse in a group is given the opportunity to fight with every other mouse in turn. This was adapted from the standard method for measuring the dominance order in a group, and usually has the effect of setting up a dominance hierarchy. The members of the group have different experiences, varying from a majority of victories to a majority of defeats. The other method is to standardize the training for all individuals in a group, either by introducing mice which are under control or by stopping the fight before serious injury takes place. In the latter case behavior is measured through response latency, or the time which it takes for a fight to develop.

The test situations themselves can be subdivided into two classes: those which are noncompetitive in the sense that the animals simply fight in bare cages and have no external object to fight for and those in which the animals compete for some object or substance. This latter situation may be further subdivided into cases in which the animals can actually get possession of some object, such as a small pellet of food, and situations in which the animal can only crowd others away from a favorable location, such as a drinking fountain or a spot in front of a food dish. As will be shown below, the differences between these situations make a great deal of difference in the results (see Table 1). For example, the noncompetitive type of fighting is usually much more vigorous, a result which provides a major experimental problem.

The early work on behavior traits (Yerkes, 1913; Utsurikawa, 1917) made considerable use of rating scales, usually based on the appearance of certain behavior patterns. This was necessary because the reaction tested was often that of savageness in response to people. In

this situation the experimenter is obviously dominant and makes attempts to provoke the subordinate rat into fighting back by restraint, teasing with a straw, etc. Such a rating scale is also useful when the amount of conflict behavior between two relatively peaceful animals is measured and only the milder forms of conflict behavior are present.

On the other hand, in the more recent experiments with fighting between mice, such rating scales have been found unnecessary. True fighting behavior is unmistakable, and its presence or absence can, in most cases, be used as the result of the experiment. Thus there is no question of validity and in most cases very little question of reliability, provided that the animals are not tested oftener than once every 48 hours on prolonged fights. More frequent bouts tend to cut down the amount of fighting, though not if the fights are terminated after a brief interval.

On the whole, the presence or absence of certain behavior traits may be considered a more objective and accurate measure of behavior than using rating scales or graded impressions. The former may be transformed into a numerical score or scale if desired, but this process must always be somewhat arbitrary.

TABLE 1

CONFLICT BEHAVIOR APPEARING IN VARIOUS SITUATIONS TO WHICH EXPERIMENTAL FACTORS MAY BE ADDED

Training	Noncompetitive	Competitive	
		Possession Possible	Possession Impossible
Primary situation (naïve animals)	Violent fighting directed toward other animal	Fighting directed toward object	Either no conflict behavior or mild pushing
Situation repeated (experienced animals)	Behavior associated with presence of other animal: dominance-submission relationship develops if fighting prolonged	Behavior associated with object: dominance-submission relationship develops if fighting prolonged	Same as above

RESULTS OF EXPERIMENTS ON INEXPERIENCED OR NAÏVE ANIMALS

At the outset, it should be stated that no animal is "inexperienced," except possibly at the instant of conception. The term is used here to refer to animals which have no known experience with agonistic behavior.

A. FIGHTING IN NONCOMPETITIVE SITUATIONS

1. *Development of fighting behavior in mice and rats.*—It is a common observation among experimenters that littermates which are raised together may live peacefully in the same box even at a quite advanced age, and Scott (1946) has found considerable difficulty in training these mice to fight. On the other hand, Fredericson has raised isolated animals (C57 black 10 strain) from the age of 21 days (1950*a*) and has found that, when they were brought together for a short period daily, all the animals had begun to fight vigorously by 36 days. The tendency to fight had appeared only 1 or 2 days

before the 36-day age, and this period in life seems to correspond to the time when the sex hormones first become active (Snell, 1941). Female mice (with a few exceptions) in this and most of the other inbred strains do not fight either at this time or later. These results support Beeman's (1947*a*) conclusion that the male sex hormone is a definite factor in causing fighting in the absence of competition.

It is also apparent that the training and previous experience of animals which have never fought have an important effect on their reactions. The results cited above in which animals raised together show a tendency not to fight may be explained as a case of passive inhibition.

However, if peaceful male mice with no previous fighting experience are attacked by others, they almost invariably fight back; and, if the mice are separated immediately so that no harm is done, they consistently fight back on subsequent occasions. Such results suggest that pain is an important factor in producing fighting in undefeated mice.

An experiment was done (Scott, 1946) on young mice by subjecting them to pain at various ages by gently squeezing the tail with forceps. The results showed that even the newborn animals reacted by squeaking and making attempts at escape. As soon as the eyes were opened, the mice would turn and bite the forceps. Two days later it was found that threatening movements of the forceps would produce the defense posture. All this occurred long before attacking behavior had been observed.

The conclusion can be drawn from the above data that there are three important factors which affect fighting in mice which have never fought before: training, the presence of the male sex hormone, and pain (which may result from an attack by another animal). Pain is obviously the most important factor in producing defensive fighting, as this type occurs very early in development and will be seen in either males or females. The male sex hormone appears to be very important in producing aggressive fighting, but its influence may be nullified by various sorts of training. Sudden or threatening movements may also be factors in inducing fighting, as restraint may be.

In rats Seward (1945) has described the development of fighting behavior in young albino rats, starting at 35 days of age. Individuals of either sex indulge in what appear to be playful wrestling matches, leaping, pouncing, and rolling over and over. Such behavior is rarely if ever seen in mice.

At about 45 days of age this playful fighting may become more serious, with squealing and defensive postures. It would appear that the start of the fight is caused by pain, perhaps as one rat plays too vigorously. It would also appear that the onset of fighting probably coincides with sexual maturation and the appearance of the sex hormones.

After puberty fights occur between females but less often lead to dominance-subordination status than in the males. Sex differences in fighting appear to be less extreme in rats than in mice.

If male rats are allowed to meet for the first time beyond the age of puberty, they apparently form dominance relationships much more quickly after the age of 100 days is reached.

In wild rats, Calhoun privately reports that the onset of lactation appears to increase the vigor of fighting in females.

O'Kelly and Steckle (1939) have reported that repeated electric shock applied to the feet of a group of six male rats had the effect of starting up violent fighting, which eventually became long-continued. Using a different strain and different apparatus, Daniel (1943) was

able to get fighting, but not of a long-continued sort. However, this provides additional evidence that pain is also in rats an initial cause of at least defensive fighting.

There have been no experiments in either mice or rats on the question of how various physical environmental factors might affect the first appearance of fighting.

2. *Conflict with people: wildness and savageness.*—As indicated above, mice and rats tend to be wild and savage when first handled by people, and this may be interpreted as a reaction to a situation of conflict with a member of a different species. Wildness includes chiefly defense and escape reactions, and savageness includes chiefly defensive fighting and possibly in some cases aggressive fighting.

No careful work has been done on the nature of the stimuli which elicit this response; as in most cases, the authors were interested in the nature of the response itself. Pain, noise, sudden movements, and restraint have been obviously involved in the experiments of Yerkes (1913), Utsurikawa (1917), and Stone (1932), on rats, and Coburn (1922) on mice; but other factors may be discovered by the use of a more-careful "releaser" technique of study.

B. FIGHTING IN COMPETITIVE SITUATIONS

Relatively few experiments have been done until recently with competition in inexperienced animals; and the most obvious fact is that the results of such experiments seem to depend upon whether or not the animal can actually get control over the object competed for. These two sorts of experiment will be discussed separately.

Bruce (1941) found that thirsty rats would struggle over a water fountain at which only one animal could drink and that hungry rats would similarly struggle for the possession of a food pellet. The behavior was not described in detail, but it is obviously not the violent type of fighting described by Seward (1945) in noncompetitive situations.

Fredericson (1950*a*, *b*) has found that mice which are not hungry will not compete or fight for a piece of food introduced into the cage. On the other hand, mice which have been starved for 24 hours or longer will struggle for a piece of food and occasionally vigorously bite or wrestle with the opponent. This has been observed as early as 29 days of age. This sort of behavior is quite different from the type of fighting found in noncompetitive situations. Effort is directed toward the possession of the food and attacks on the opponent stop as soon as the food is gotten. This contrasts with the much more vigorous and violent type of fighting which occurs in the absence of competition, where activity is directed entirely toward injuring or escaping from the opponent and fighting goes on for long periods. Furthermore, the competitive type of fighting is independent of the male sex hormone, since it can be produced in females and in males as young as 21 days of age.

This new technique opens up the possibility for experiments in which other types of things can be competed for, as, for example, the possession of a female in heat. Though dependent upon the male hormone, the noncompetitive type of fighting may take place whether or not the female is present or receptive.

A reference may now be made to situations in which complete possession of the object competed for is not possible. Ginsburg and Allee (1942) found no effect of starvation up to 36 hours (it is not stated whether or not food could be possessed). Mice thirsty for 6–8 hours did not fight over a water bottle with a small nozzle. In rats Bruce (1941) found that thirsty

animals which were running in an alley showed no competitive behavior over drinking at a fountain if it could be shared, and Fredericson (1946) found not only mild pushing but some indications of dominance where hungry rats were given a food cup which permitted only one animal to eat at a time.

Seward (1945) performed experiments with both types of situations, using pellets and a food-hole. In both cases the rats had previously established a dominance order and were allowed to compete for food later. The amount of fighting was about the same with the pellet (which could be possessed) and was markedly reduced with the food-hole. These results are discussed later in connection with trained animals.

The evidence is not entirely clear, but the following tentative conclusions may be drawn: (1) in both rats and mice, hungry animals will struggle for the possession of a food pellet, but with considerably less violence than in noncompetitive fighting; (2) thirsty rats (but not mice) will struggle for a place at a water fountain; (3) in rats, a situation such as a food-hole, in which one animal can block off another from food, tends to produce less fighting than a pellet which can be possessed. The inconsistencies may be caused by hereditary differences between rats and mice but are more likely caused by differences in technique. The amount of struggling would appear to be proportional to its usefulness in the situation.

To summarize this section on fighting in inexperienced animals: the importance of training and experience is clearly indicated, as well as the importance of the type of situation to which the animals are exposed. Factors which are important causes for fighting in one situation are ineffective in others. In noncompetitive situations the important factors besides early training are age, the male hormone, pain, threatening movements, and possibly noise. In competitive situations, where possession of a portable food object is possible, hunger may be added, the male hormone is unimportant, and pain and threatening movements do not seem to have much significance.

One serious question raised by these results is the reason for the existence of noncompetitive fighting. In mice raised in cages it serves no obviously useful purpose, and, if such noncompetitive fighting appears under natural conditions, it would seem to be a disadvantage to the species. Two suggestions may be offered here. One is the possibility that it tends to keep a population so scattered that a group of males cannot be concentrated around a food supply in a given area (there are no indications that mice ever defend a definite territory as birds do, although Calhoun finds indications of this in wild rats). The other suggestion, which is based on certain observations to the effect that fights may start as the result of attempts by one male sexually to approach another, is that the fighting may serve to prevent sterile sexual activity. The confirmation of these hypotheses must await the results of experiments on behavior in relation to socially organized populations.[4]

The data also bring into question the universality of the frustration-aggression hypothesis. Under certain conditions when the possession of an object is the problem, frustration leads to aggression in mice and rats. When possession of the object is impossible, frustration does not necessarily lead to aggression. Furthermore, in noncompetitive fighting there is no obvious way in which the animals seem to be frustrated, unless it may be

[4] Calhoun suggests from his observations that in "noncompetitive" fighting, rats and mice fight for undisturbed possession of living space, rather than any particular bit of space. The result would be dispersal of the population.

the frustration of a sexual appetite. Experiments in this area which involve sexually satiated animals would seem to be indicated; but, on the whole, it may be said that frustration leads to aggression where aggression provides the solution to a problem (i.e., satisfactory adjustment).

C. NEEDED EXPERIMENTAL WORK

It will be obvious from the above account that almost no work has been done with factors which might be expected to cause inexperienced animals to start fighting. Males seem to start their noncompetitive fighting at about the time when the male sex hormones might be expected to appear first, but there has been no experimental analysis to see whether this could be produced at earlier ages. Experimental work also needs to be done on the effect of lactation and the various endocrine changes which go with it.

Little work has been done on the effect of environmental factors which might cause inexperienced males living together to start fighting. Littermates brought up together and living undisturbed in the same cage usually do not start fighting until well along into maturity, if at all; and it would be interesting to see whether or not the stresses of cold, hunger, sexual frustration, etc., might cause such males to initiate fighting. Once the habit is established, of course, these factors would probably become unimportant.

With regard to fighting in competitive situations, experiments have been done only with food and water, and the conclusion seems to be that fighting results only when possession of the object can be assured. So far there is no indication that competition for a sexual object will cause fighting in rats or mice. Ginsburg and Allee (1942) found that when one of two nonaggressive males is placed with a female in estrus, allowed to copulate, and then is placed with the other male, the latter pursues the first and may start a fight. This appears to be a case where one male reacts toward another as if it were a female, and the resulting homosexual behavior leads to a fight, rather than a case of competition for possession of a female. Further experiments are needed along these lines.

RESULTS OF EXPERIMENTS WITH EXPERIENCED OR TRAINED ANIMALS

Experiments of this sort have been designed to test the effect of various factors upon the behavior of animals which are used to fighting. Experimental factors might therefore be expected either to reduce or to increase the usual amount of agonistic behavior. Theoretically, three types of situation might be employed: one in which two animals had been allowed to develop a relationship of dominance and subordination; one in which animals have been trained to fight, but not allowed to develop dominance; and, finally, one in which the two animals have been trained to be peaceful. The first of these has been used extensively with noncompetitive fighting, the second with competitive fighting, but the use of the third has been little explored.

A. FIGHTING IN NONCOMPETITIVE SITUATIONS

Most of the work which has been done in this area has been directed toward modification of the dominance-subordination relationship, which has been previously defined. An extensive and thorough series of researches on mice has been done in this field by Beeman and Allee, who have used the technique of round-robin encounters, in which a small group of mice, isolated except during the experimental period, are fought at regu-

lar intervals in rotation. After a few encounters, dominance relationships of a fairly stable sort are set up and other factors can be introduced. In such a technique the usual result is to place one animal on the top and another on the bottom, but the intermediate animals may not be clearly ranked among themselves, probably because mice appear to have difficulty in recognizing other individuals except by their attitudes of attack or defense.

1. *Ecological factors* (*physical*).—Beeman has privately reported no effect of cold on the incidence and vigor of fighting. Males were kept at a temperature of 17° C. for 24 days before round-robin encounters were begun. Scott and Marston (1946) did an experiment in which pairs of male C57 black mice which had established dominance were alternately fought at a temperature of 50° F. (approximately 16° C.) and at room temperature (70° F.). No significant difference was observed, although there were some indications that the low temperature might slightly lower the amount of fighting. This is contrary to casual observations made on people, who appear more irritable when exposed to sudden temperature changes, and it may be explained by the fact that adjustment to temperature in mice is probably a matter of thyroid adjustment rather than changes in circulation, so that no immediate physiological effect would result from a sudden change in temperature. Thus it would appear that neither long exposure nor sudden change to low temperature has any appreciable effect on the habitual fighting of mice.

On the other hand, Ginsburg and Allee (1942) and most other experimenters have observed that mice which have been exposed to high temperatures tend to fight less vigorously and attempt to adjust by digging in shavings or flattening themselves out on a cool surface. In the absence of detailed experimental work, it may be tentatively concluded that high temperatures have an inhibiting effect on the amount of general activity.

The possibility exists that mice may fight over nests, although this has not been observed. Scott (1944*b*) reported that males were never found in nests with females and very young mice when the animals were given the opportunity to construct substantial nests. However, the females were never observed in the act of driving them away.

A certain number of experiments have been done with the factor of space. In the usual laboratory set-up, mice or rats are kept in isolated cages and the usual experiment involves transferring the mice to another cage or to that of another mouse. Uhrich (1938) reported that mice left in their home cages were likely to have more success in fighting than were introduced mice. The observation of such mice indicates that the stranger spends most of his time investigating the pen, while the home mouse concentrates on fighting. The effect might be due either to the defense of the home cage or to the fact that the strange mouse is upset and nervous.

There appears to be no evidence for the guardianship of specific territories in mice. Using a multiple pen with nest boxes connected by runways, Scott (1944*b*) was unable to find any indication of territorial defense. However, such a possibility cannot be excluded, if more space were given. Calhoun (1949) has observed cases in rats living under crowded conditions but in a large field, where certain animals appeared to be confined to certain areas by the attacks of others, the dominant animals ranging more widely. The result is a rather vague sort of territoriality, in which most animals are excluded from a given area where one or

more dominant animals live. There appeared to be no cases in which definite boundary lines were defended, as reported in birds, where one animal is dominant in his own territory and subordinate in another's. Such activities would be very difficult in mice and rats, which are largely nocturnal in habit and accustomed to living in areas which provide a great deal of cover.

Crew and Mirskaia (1931) have reported experiments in which space was kept constant, but the density of animals varied. Varying numbers of pairs of mice were placed in small cages of uniform size. Dominance was not recorded, but the maximum death rate (50 per cent) was observed in cages containing 4 pairs of mice, with slightly more favorable results with either larger or smaller populations.

The results must be interpreted with regard to density rather than to space. Crowding tends to increase the probability of fighting but, beyond a certain point, exerts a favorable effect on mortality by permitting the beaten animals to hide and escape notice. However, it may be inferred that small space tends to prevent the formation of a dominance order, which would permit survival of individuals.

Scott (1946) experimented with dominance in two types of pens, one large and one small, and reported that behavior between dominant and subordinate animals was significantly altered. In the large pens the dominant animal did more chasing and less attacking, while the subordinate animal did more running away and adopted the defense posture fewer times. This indicates the adaptive nature of these responses.

In summary, heat and probably cold tend to reduce the amount of fighting behavior. The factor of space has been definitely shown to have an important effect on the dominance-subordination relationship by modifying the type of agonistic behavior and upon social organization by allowing a vague sort of territorialism in large areas.

2. *Ecological factors (biological).*—No work has been done with biological factors in the environment with the exception of using the behavior of one animal to modify that of another. For example, a severe defeat will cause a dominant animal to become subordinate not only to the aggressor but also to other animals which he meets soon thereafter (Ginsburg and Allee, 1942). Conversely, Scott (1944*a*) found that a habitually peaceful relationship could be upset by training one of the animals to fight. Thus, while there are indications that the biological environment may be important, the nature of the factors is really social and psychological and should be discussed under the section on training below.

The effect of predators and parasites on fighting behavior has not been studied.

3. *Physiological factors.*—The effect of nutrition and hunger on fighting of animals which have set up a system of dominance has been studied extensively. Ginsburg and Allee (1942) found that hunger and thirst did not affect the tendency to fight. Seward (1945) performed extensive experiments with hungry rats in which he used situations in which the animals could obtain possession of the food and could not obtain possession of it. The result was to cut down the amount of habitual fighting, particularly in the case where possession was impossible.

Seward used these results to question the frustration-aggression hypothesis, arguing that the hungry animals should have become more aggressive because they were frustrated. However, since training seems to be the all-important guiding factor in fighting, it will be seen

that in his experiment he simply tried to introduce a new type of countertraining and stimulation, which tended to interfere with habits already formed. A fair test of the frustration-aggression hypothesis would be to take mice or rats which had been trained in a competitive situation and then vary the amount of frustration. Such an experiment would be comparable to those in goats (Scott, 1948) where positive results have been obtained. However, all these results tend to indicate that *frustration as a cause of aggression is only important where animals have been trained to react aggressively after frustration.*

Beeman and Allee (1945) have done a series of experiments in which Vitamin B_1 deficiency was used as an experimental factor. It was found that, once dominance had been established, the dominant animal could maintain his position almost to the point of death from avitaminosis, the subordinate animal still responding to the threatening attitude of the feeble dominant male. It is probable, however, that the amount and vigor of fighting was affected, even though the number of fights won was not.

From these experiments it may be concluded that nutritional deficiencies may lower the amount of fighting, either by distracting the animal's attention, as in Seward's rats, or by causing a diminution of vigor, as in Beeman's mice, but that the dominance order and habit of fighting are very little affected.

Beeman (1947*a*, *b*) has also done some excellent work on the effect of the sex hormones on fighting. Females in the domestic strains of mice very seldom get into fights in the ordinary situations to which they are exposed, whereas fighting in males is much more common. She therefore castrated male mice at various ages and allowed them to rest 25 days before starting to train them to fight, using the round-robin technique. These animals never fought. Replacement therapy with testosterone propionate pellets was followed by the development of the usual amount of aggressiveness in the mice, and removal of the hormone pellets caused an almost immediate cessation of aggressiveness.

However, the fact that some animals continued to fight led her to make further experiments in which animals were first trained to fight and become dominant in the round-robin situation and then were castrated and tested for fighting without being given any rest. These animals continued to fight without interruption. These results may be explained either as the continued effect of strong habit, or it may have been that, once the habit of fighting has been established, the small amount of male hormone from the adrenal gland is sufficient to maintain aggressive activity. Beeman is continuing work to test the adrenal gland hypothesis at the present time.

The conclusion must be reached that the presence or absence of the male hormone has a very important effect, at least on the initiation of fighting under noncompetitive situations. Whether or not it has an effect on a well-established habit of fighting is still in doubt.

Beeman has also started an experimental program on the effect on fighting produced by stopping the function of various sense organs and by ablation of various parts of the brain; but this work cannot be reported on as yet.

It must be concluded that, once habits of fighting or running away have been strongly established, organic changes have very little effect unless they are so serious as to threaten the life or cripple the functions of the animal. The one possible exception to this is the male hormone, and even it seems to have much more effect on the initiation of fighting

than upon strongly formed habits of fighting. Since pain (as pointed out in an earlier section) has the effect of causing young females, as well as males, to bite back, the hypothesis may be tentatively suggested that the hormone has the effect of lowering the threshold of sensitivity to painful stimuli. This fits in very well with the time in development in which noncompetitive fighting appears.

It is obvious that many more experiments remain to be done in this field. In evaluating experiments of this type it is essential (because of the importance of training) to keep in mind the point as to whether or not the experimental factor was altered before or after the training period began, and the amounts of aggressive or escape behavior should be measured as well as dominance.

B. COMPETITIVE SITUATIONS

The only work in pure competitive situations which has been done on mice is that of Fredericson (1950*a*), who has repeatedly allowed hungry mice to compete for food, separating them before dominance develops. After a period of training in the competitive situation the animals will compete for food, even though they are not hungry, indicating that frustration is not necessary, once the habit of fighting has been built up. However, Fredericson has done no work as yet on the decrease or increase in fighting as a result of frustration, and in no case has he worked with situations in which dominance has been developed.

Obviously, the situation has not been so thoroughly explored as has dominance in the noncompetitive situations; but here again the tentative conclusion can be made that training is a very important factor compared to physiological variables. Females respond in this situation as well as males (Fredericson, 1951).

EXPERIMENTS WITH TRAINING

A. EFFECTS OF EARLY ENVIRONMENT

Kahn (1949) has found that mice which are severely defeated at various immature ages will less readily attack dangled mice when given the opportunity as adults than will adults with no such previous experience.

Fredericson (1950*b*) has shown a similar effect with competitive fighting. Young hungry mice between 29 and 35 days of age were allowed to compete for food and were later tested in a nonhungry state at 72 days of age. They immediately competed for food, while control animals did not. Even a single experience appears to produce this effect.

As pointed out under the section on development of fighting behavior, Fredericson has found that male mice which are raised alone and periodically allowed to investigate each other for short periods will begin to fight at around the age of 36 days. On the other hand, it is commonly observed that males which are raised together in the same cage and left undisturbed often do not fight even after they reach maturity.

The fact that animals which are raised together do not fight may be explained by the hypothesis of passive inhibition. In Pavlovian conditioning the repetition of a stimulus to which there is no primary reaction will cause that stimulus to be associated with lack of activity; and when it and a stimulus which is associated with activity are presented together it can be shown to have a definite inhibiting effect. Presumably the young mice raised together which do not fight up to 30 days form a strong habit of not fighting, which is sufficient to overcome stimulation which under other conditions may produce it. Thus there is good evidence that early training and experience, even though fighting may not be in-

volved, has an important effect upon fighting in later life.

B. THE PRINCIPLES OF LEARNING

As intimated in the preceding paragraph, the effects of experience on fighting behavior can be related to the vast body of knowledge which psychologists have collected under the heading of learning phenomena.

These data need be treated only very briefly here. The field has been very adequately summarized by Hilgard and Marquis (1940) and by Hilgard (1948). The pioneer work of Thorndyke and Pavlov has been expanded by such workers as Guthrie (1935), Hull (1943), and Skinner (1938), so that certain general principles have been established. For the most part these may be termed "laws," in the sense that they are descriptions of behavior rather than causal explanations. The most important of these may be summarized as follows:

1. *Adjustment or adaptation.*—All behavior is an attempt to adjust or adapt to change, whether the changes are internal or external. As a corollary of this, it may be said that behavior is not a response to a condition but to a change in conditions.

2. *Primary stimulation or releasers.*—Certain responses are automatically given to rather specific stimuli. Such responses form the raw material of learning, usually appearing early in development.

3. *Association, reinforcement (memory).*—If a change to which no response is automatically given (secondary stimulus) is repeated together with a primary stimulus, the response may thereafter be given to the secondary stimulus alone (association). The repetition of the primary and secondary stimuli together is called "reinforcement."

4. *Negative association (inhibition, extinction).*—If a secondary stimulus is repeated in the absence of a primary stimulus, it will tend to be associated with a lack of response. Such a stimulus given together with a secondary stimulus which is associated with a response will tend to weaken the response (inhibition). If a secondary stimulus associated with a response is repeated without reinforcement by a primary stimulus, the response will tend to die out (extinction). It should be pointed out here that a secondary stimulus may be associated with a lack of response either before (passive inhibition) or after reinforcement has taken place—a concept which is of considerable importance in the control of fighting.

5. *Strength of association (memory).*—The immediacy with which a response is given to a secondary stimulus or the number of times it will be repeated without reinforcement is determined by the manner in which reinforcement is given. Primacy, recency, and frequency of reinforcement appear to be particularly important. Once reinforced, an association will reappear after extinction, provided that sufficient rest is given, indicating that the process of association is a permanent one.

6. *Generalization and discrimination.*—When an association has been built up between a stimulus and a response, there is a tendency to respond to all similar stimuli (generalization). Responses to unreinforced stimuli are eliminated or extinguished by negative association (discrimination).

Several basic research problems arise out of these laws. The last four are essentially based on Pavlov's work with conditioned reflexes. Can the interaction of these laws explain the results of experiments on complex learning and problem-solving? A major controversy in psychology is raging about this point, the Gestalt psychologists and such leaders as Tol-

man (1932) pointing out that the organizing capacity of the nervous system may lead to results which cannot be explained by a knowledge of simple elemental processes. However, this controversy has little bearing on the causes of fighting in rats and mice, since in most cases no complex problem-solving situations are involved.

A second major question is whether or not these laws are applicable to all species of animals. At the present time it may be safely said that the first two apply to all species of animals and that the last four, which are chiefly laws of learning, apply to all vertebrates which have been tested. Whether or not the process of learning in invertebrates is essentially different has not been established. All these laws should apply to rats and mice, and there are many confirmatory experiments on rats.

The third problem is whether or not these principles can be extended from reflex to voluntary behavior, and from these to complex patterns of social behavior. The work of Skinner (1938) on what he has called "operant conditioning" has abundantly confirmed the extension of these laws to voluntary behavior. In his experiments the primary stimulus is food, and the primary response is eating. However, in order to get food, the experimental animal must do some voluntary act, such as pressing a lever, which may be called a "secondary response." This becomes associated with eating; and it is clear that Skinner is working with the association of two responses (both voluntary) rather than with association of a stimulus and response.

The laws of association, negative association, and strength of association are abundantly confirmed; and, although the experimental technique does not lend itself so easily to the study of generalization and discrimination, it would appear that these also apply.

The final question of whether these laws apply to social behavior has been most thoroughly tested on the fighting behavior of rats and mice, with confirmatory results which will be discussed in the next section.

C. TRAINING ANIMALS TO BE DOMINANT AND SUBORDINATE

Experiments with dominance in mice have been done almost entirely in the noncompetitive situation, but, before this is discussed in detail, it is essential that the term be thoroughly understood. As defined at the beginning of this article, a relationship of dominance and subordination is one in which one of two animals regularly fights or exhibits aggressive behavior and the other regularly exhibits escape or defensive behavior. It should be emphasized that the dominance relationship is one which involves true agonistic behavior of some sort.

From a theoretical point of view it may be assumed that the relationship is caused by simple conditioning, the dominant animal having formed the habit of attack and the subordinate animal having formed the habit of escape or defense (Ginsburg and Allee, 1942).

Since experience or training is assumed to be the cause of dominance and subordination, it would, of course, follow that the previous experience of the animal would be the all-important factor. This expectation was brilliantly and thoroughly confirmed by the work of Uhrich (1940), and Ginsburg and Allee (1942), who first showed that previous success tends to produce a dominant animal, whereas previous defeat tends to produce a subordinate animal. They also found that these roles could be reversed. A succession of bad defeats will cause a dominant animal to become subordinate.

With much more difficulty, involving a long period of rest and a series of easy victories, a subordinate animal can be trained to be dominant.

Considered in detail, the experiments of Ginsburg and Allee are seen to have a direct bearing on the laws of learning. The authors first established the fact that three inbred strains of mice differed in fighting ability, so that one could be depended upon to defeat another in an experimental situation. Using the round-robin technique, groups of mice in each strain were fought in neutral cages until a dominance order was established in each. These processes may now be analyzed from a theoretical viewpoint.

There is some doubt as to what may be the primary stimuli that evoke fighting behavior. Certainly, one of these is slight pain; another may be the mere sight or contact with a strange male mouse. The sight of an animal running away also appears to be effective. On the other hand, defensive and escape behavior are evoked by *intense* pain. Once a fight is started, the two animals reinforce each other's fighting by mutual injury, until one may be badly hurt and respond with escape behavior. The victor keeps attacking, reinforcing escape behavior with further injury. If this situation is repeated, reinforcement of fighting occurs in one mouse, which becomes dominant, and reinforcement of escape or defensive posture occurs in the other, which becomes subordinate. Meanwhile, secondary stimuli become associated with the response. The change to the fighting cage results in preliminary fighting behavior by the dominant animal, and passivity by the subordinate one.

It may be pointed out that the establishment of dominance tends to lessen reinforcement of both animals. The dominant animal is not hurt so much and should not fight so hard; if the subordinate one escapes, it should not be hurt and should not attempt to escape so hard. Such lessening of vigor of fighting is commonly observed where contacts are repeated after dominance is established.

A pair of mice which have been trained to fight in this way will both have some reinforcement of fighting behavior. Therefore, one might expect that the subordinate mouse, after a long period with no reinforcement, would revolt and fight again and that a dominant mouse recently defeated would continue escape behavior because of recency of reinforcement. Such appears to be the case.

To take an example, Ginsburg and Allee took an albino mouse (strain C) which stood at the top of a group of 4 other albinos and subjected it to severe defeats twice a day for 8 successive days by the alpha mouse of the C57 black group. The dominant albino fought back only during the first three battles. The next day he was matched against all members of the albino group three times. On the first round he was completely submissive to all mice (showing escape or defense reactions) but was not completely submissive by the third. On the next day, during the third round of fights, he attacked one of the white mice, won, and thereafter assumed the dominant position in the group. When exposed to nonagressive black and agouti animals at this point, he was submissive.

All this can be interpreted as reinforcement of fighting, extinction of fighting, and reinforcement of defensive behavior while being beaten by the black, generalization of defensive behavior toward all animals, including the whites, and, finally, discrimination between them and colored animals. The only unusual feature seen in applying the principles of learning to fighting behavior is that the reinforcing stimulus for fighting is the

same as that for escape behavior, except in degree, and that therefore extinction of one response necessarily results in reinforcement of the other.

The lowest-ranking black mouse, which did not fight at all, was isolated for 5 weeks (neither reinforcement nor extinction of fighting). He was then matched with a submissive mouse, which he attacked after it had reacted defensively to grooming and had run away. Afterward, he was able to beat all the other black mice except the dominant mouse; but, when he was returned to the round-robin system of fighting, he gradually went back to his former place in the dominance hierarchy.

The reappearance of fighting after a rest is strikingly similar to Pavlov's description of the revival of a conditioned reflex following extinction, provided that rest is given. Fighting was reinforced in bouts with weaker mice for a short period but was later extinguished, and escape behavior was reinforced by encounters with a superior male. The only puzzling thing here is why he was unable to discriminate between aggressive and submissive black mice; but, since the members of an inbred strain are strikingly similar in appearance, this may not be too surprising.

Ginsburg and Allee's results were empirically obtained from an experiment designed to test the results of defeat and victory, and the resemblance to Pavlov's results was noticed afterward. Seward (1945) afterward made a deliberate attempt to see whether Pavlovian effects could be predictably obtained on a group of rats. The principal differences were that he used a group of albinos of undetermined inbreeding and that he included both females and males. Moreover, he used more elaborate measures of behavior in addition to simple dominance and submission.

The first thing that he obtained was a striking confirmation of the predicted effect of lessening of fighting because of lack of reinforcement as dominance was established. In the males the mean number of "aggressions" (pushing, mauling, nipping, etc.) per bout fell from 24.0 in the first round to 2.5 in the eighth, while in females it fell from 23.9 in the first to 11.2 in the fourth, after which observations were not continued. Other indexes of the amount of fighting activity showed similar drops.

Next, Seward was able to repeat Ginsburg and Allee's results with defeated animals. Rats which had been beaten once rose to the original level of aggressions per bout after 14 exposures to nonaggressive animals. However, beaten animals which had been isolated 1 week showed no rise in aggressiveness, indicating that the rise in aggressiveness was a real learning phenomenon (extinction) rather than the effect of disuse.

Using the same group of rats, Seward fought 10 pairs together, then fought the winners together, then matched them again in the original pairs repeatedly. The result again confirmed the expectation that repeated matching of a pair together should lead to lessened activity because of lack of reinforcement. The winners tended to become less aggressive, and the losers more aggressive. Seward, however, observed a special effect on the winning rats, after the second fight (not with the usual partner), in that they showed symptoms of fear and appeared to be in an unstable balance between fear and aggression, presumably as the result of having had a hard fight.

The chief difference between these results and those of Ginsburg and Allee is that no evidence of spontaneous recovery of aggressiveness or of escape behavior following disuse is found. Further experiments, dealing with short rest periods of

24 hours or longer ones up to approximately 1 week gave the same result. However, in the mouse experiments, much longer periods of isolation were used, which may explain the discrepancies.

These theoretical details may obscure the very important practical aspect of Ginsburg and Allee's (1942) work, that motivation for fighting is increased by training in accordance with reinforcement theory; i.e., a tendency to fight is increased by success in fighting. This was confirmed by Fredericson (1949), using a different technique. Pairs of mice in adjoining cages were let in together once per day and were separated 10 seconds after fighting began. The response latency, or time at which the fight started, went down very rapidly and reached a stable level after the third trial.

Taken together, the researches of Ginsburg and Allee and those of Seward form a major body of evidence that the principles of associative learning apply to a major type of social behavior, as well as to simple reflexes and learning tasks. Further, at least some of the principles of more complex learning, such as problem-solving, also apply to agonistic behavior, as shown in the work on taming discussed below.

It must be emphasized that the experiments on dominance in mice have been done under very artificial conditions, in small cages and often allowing the animals to meet only at the time when the fight takes place. What sort of relationship would be developed under more natural conditions where the defeated mouse can escape cannot be predicted.

Calhoun (1948) has recently made studies of rat behavior under seminatural conditions but with the restriction that the defeated animals could not completely escape from the situation, the whole area being fenced. He finds strong evidence for dominance and subordination starting in early life when younger animals are given light beatings by the older ones. One of the chief results is to prevent the subordinate animals from having an undisturbed home range. Because of constant disturbance, the subordinate animals grow and reproduce less successfully.

One interesting peculiarity of dominance in caged rats and mice is that in noncompetitive fighting the animals tend to be either dominant over all the members of a group or, if not, subordinate toward all other animals. This was originally discovered by Uhrich (1938) in his studies of caged groups of mice and tends also to come out in work carried on in the round-robin method. This contrasts markedly with the results in chickens, goats, etc., where there is a tendency for a straight-line system to be developed within a group, in which the intermediate members are dominant over some animals and subordinate to others. It also contrasts with Calhoun's (1948) results with rats in large spaces.

The precise explanation cannot be given at the present time, but two possible explanations have been suggested. One is that the animals cannot recognize other individuals except by their attitudes and behavior, which indicate either dominance or subordination. Inbred strains have been largely used in these experiments, which would make individual recognition difficult in any case. Another possibility is that the fierceness of the fighting is so great that an animal is either victorious or so soundly beaten and emotionally disturbed that he has no chance to discriminate when he meets another individual. A third possibility is that this result is caused in some way by the limitations of a cage environment.

It will be interesting to see whether or not the milder type of fighting which

is associated with competition will lead to a different sort of dominance organization.

D. TRAINING ANIMALS TO FIGHT, TO RUN AWAY, OR TO BE PEACEFUL

For the practical purposes of research, Scott (1946) has developed systems for training animals to fight or be peaceful, based on general theoretical principles. These will first be considered in connection with the problem of producing a vigorous fighter: (1) To train an animal to fight, he must be affected by a primary stimulus or releaser, the easiest one of which to use is slight pain. (2) The fighter must be stimulated by a variety of methods, or by different animals which behave differently, as it has been observed that when the same method or animals are used over and over again the intensity of fighting tends to diminish. This may be explained by the fact that a stimulus is a change and that a repeated stimulus is less of a change and, furthermore, permits the animals to adjust to it with decreasing effort. (3) Animals become fatigued after violent fights and do not usually fight vigorously if they do it every day or more frequently. (4) The animals also fight more vigorously if they have a sort of warmup period and are thoroughly excited before the actual fight starts. (5) The fighter must be successful in the fight and not be beaten, as defeat teaches it to run away rather than to fight back. Repeated success leads to strong motivation for fighting, whereas repeated defeat produces a strong habit of escape (see Ginsburg and Allee, 1942).

Using all these principles, the following practical method was devised for developing a vigorous, persistent, and successful fighter. The mouse is first attacked by a trained fighter to which he will normally respond by fighting back. The two animals are separated before any damage occurs (if no trained fighter is available, the dangling procedure described below is almost as effective). On the second day a helpless mouse is dangled in front of the fighter and bumped against him gently in such a way that the fighter is encouraged to attack. Four different mice are used in this way, the second and fourth being turned loose in the pen for short periods. The helpless mice are not used long enough to be badly hurt. This procedure is repeated daily, and on the fifth day dangling is followed by releasing an untrained, strange male in the pen. The trained fighter will now attack and in almost every case will win the fight without difficulty. After this time, the trained fighter is given a warmup period before each fight but no further training is necessary. He should not be fought oftener than every other day, and longer periods of rest will not be harmful.

Fighters trained in this fashion become so ferocious that, as Kahn (1949) has shown, they will attack females and infant mice, which they would ordinarily leave alone. Kahn's experiments seem to show that they attack the juvenile mice even more vigorously than the older animals, probably because the young ones give more violent escape reactions. Another interesting fact is that fighters trained in this way which have been uniformly successful show little, if any, preliminary emotional reactions but attack quickly, savagely, and efficiently (Scott, 1947).

Using such animals as these, it is easy to train naïve animals to respond habitually to a conflict situation with escape or defensive behavior. A naïve animal is placed with the trained fighter, who has considerable advantage and usually wins the first fight. The defeated animal usually ceases all resistance after two daily 30-minute periods and thereafter runs

away at the sight of the trained fighter or, indeed, of any other mouse which is seen under the conditions of the experiment.

The same principles may be used in reverse in order to train naïve animals to develop peaceful relationships (Scott, 1944*a*). There the principal problem is to avoid or overcome the effect of the primary stimuli or releasers. After this, passive inhibition will do the rest. Male mice are placed with females with whom they ordinarily do not fight. Then, on four or five successive tests the males are taken out and given rough handling, i.e., are held by the tail with the forceps, placed on a wire screen and firmly stroked with the finger. This has the effect of giving the animals slight pain, but they are restrained from biting or effectively attacking. As soon as the handling is over, they are placed back with the females, with whom they are already used to not fighting. Considering this treatment as conditioning, the animals are learning to associate slight pain (normally the primary stimulus for fighting) and the conditions associated with it, with not fighting.

If two males which have been treated in this way for 4 or 5 successive days are then placed together in a pen with one of the females, no fighting results, either at the time or for as long as several weeks afterward, which was the period of observation. Presumably, the longer this peaceful relationship lasts, the stronger the habit of not fighting becomes. In short, if animals can be prevented from fighting at the first meeting, the principle of passive inhibition will tend to keep them from fighting subsequently.

Such animals can be *trained* to fight subsequently, using the methods described above; but no effort has been made to measure whether or not fighting is as severe. Presumably, it would be somewhat less severe. Neither has any experiment been done with training trained fighters to be peaceful, but presumably this could be done, using the same methods but with the expectation that a much longer period of training in restraint would be required than with inexperienced animals.

E. TRAINING ANIMALS TO BE TAME

It is the common experience of experimenters that repeated handling results in gradually decreasing vigor of response, so that the animals may in a short time give little or no reaction and may be said to be "tame." This may be interpreted as adaptive behavior resulting from the solution to a problem. Escape and defensive behavior are not effective and may even result in the animal's hurting himself. The ineffective pattern of behavior is therefore abandoned in favor of one which results in good adjustment, namely, a passive attitude on the part of the animal toward the experimenter.

One of the best experiments of this sort was done by Stone (1932), who tested several genetically different groups of rats and rated them for wildness and savageness. The rates for all groups descend in what appear to be fairly typical learning curves, and rats in "wild" and "tame" strains are much more closely alike in the end than in the beginning. Similar results were obtained by Coburn (1922) with mice, and, indeed, the same results have been obtained by any experimenter who has tried repeated tests. It may thus be tentatively concluded that principles of complex learning apply to agonistic behavior.

EXPERIMENTS WITH HEREDITY

A. THEORETICAL EXPECTATIONS

The principles of transmission and physiology of hereditary factors have been so thoroughly worked out in relation to various morphological and physi-

ological traits that it is possible to predict with considerable certainty the results of genetic studies of agonistic behavior.

1. Since even simple characters are affected by many genes (at least 17 known genes affect coat color in the mouse) and since behavior traits involve activity of the whole body, it is almost a foregone conclusion that any hereditary variability of agonistic behavior will be caused by multiple genetic factors and that variability in a random-bred population will fall into a normal curve.

2. Since the great majority of mutant genes are recessive to their normal or wild-type allelomorphs, it would be expected that crosses between animals showing "wild" and "tame" behavior characteristics would show dominance of the "wild" kind of behavior in the first generation. Where crosses are made between two "tame" types, the effect of the multiple factors would probably be intermediate in the first generation.

3. Since many genes are known to have effects on many characters, the possibility that known genes whose major effect is on other characteristics may also affect behavior must be kept in mind. However, such effects would be expected to vary in different genic backgrounds.

4. Since behavior consists of responses to and adaptation to environmental changes, it will be expected that under most situations environmentally caused variability in behavior will be larger than genetically caused variability. This does not modify the possibility that genetic variability may be very important under special conditions, either because of thresholds or because of uniform environmental conditions.

B. METHODS AND EVIDENCE

The experimental evidence may now be examined to see how far the use of various techniques supports or denies these expectations. To begin with, a behavior trait is probably one of the least favorable for the analysis of a precise mode of inheritance, first because the measurement of behavior traits is always time-consuming and may take anywhere from several minutes to even hours on one individual and, second, because (since all genetic experiments depend upon the analysis of variability) large numbers are always needed. The thorough study of the inheritance of a behavior trait, therefore, becomes an extremely laborious procedure; and, before such studies are made, everything possible should be done to cut down the work, and serious consideration should be given to the question of whether the experiment needs to be done at all.

One way in which time can be saved is to perfect simple and brief tests of behavior, so that each individual can be classified in a short period of time. Great care should also be taken to cut down variability by standardizing the environment and care of the animals as closely as possible. The more the environmentally caused variability can be reduced, the easier it will be to study the genetic variability.

It should be remembered that the basis of all genetic experiments is the control of genetic variability, and this means that the parental stocks must be thoroughly known from the genetic point of view. Maximum control over variability is achieved by the use of highly inbred strains of animals; and, in making an experiment involving fighting behavior, the first step would be to make a brief survey of many inbred strains and select two which have the desired contrasting characteristics. For the purpose of genetic analysis the best strains would be those which showed small variability within

themselves and large differences between the strains.

The most highly controlled experiment of all is one which involves a mutation within an inbred strain. The mutant gene can be kept heterozygous while close inbreeding is continued, so that the only genetic variability in the experiment is that caused by the gene.

If only partially inbred strains are available, such as colony-bred animals which have been kept a long time without outbreeding, variability can be somewhat reduced by using a small sample from each colony, such as taking all parent-animals from a single litter. The control of heredity achieved here will, of course, be much less; and, unless the two colonies are known to be widely and distinctly different, cross-breeding experiments are probably not worth while.

Another method which is sometimes used for study of hereditary factors is that of selection of individuals into strains according to the traits which they exhibit. Since the behavior trait may be influenced by many genes, selection without close inbreeding may result in selection of many genetic types of individuals which appear similar in behavior, and it would be very likely that there would be very little control over variability and hence that cross-breeding experiments would be largely worthless. However, the degree of separation between the two strains gives a good measure of the amount of hereditary variability in the original stock.

Several studies which have been done on this subject may now be discussed in the light of the above considerations. In the first place, important data may be obtained without using cross-breeding experiments, and the most time-saving technique is that suggested by Keeler (1942, 1945) and Keeler and King (1942) of studying effects on behavior of mutant genes which occur in inbred stocks and which affect morphological characteristics. The mode of inheritance of these genes can be easily worked out; and, if the stock is known to be pure and uncontaminated, it is very unlikely that a separate mutation affecting behavior would occur at the same time. Keeler has made a survey of mutant strains derived from a *partially* inbred strain of wild-type Norway rats and finds wide differences in behavior traits. He particularly stresses the association of tameness with the color black. These results are unfortunately not conclusive, since the original strain was not highly inbred and the differences in behavior can be accounted for by chance selection of other variable genes.

As a minor piece of negative evidence, in making a survey of differences in aggressiveness in mice, Scott (1942) observed a mutant which had originated in one of the highly inbred C57 black strains (C57 leaden) and found no appreciable differences between the original strain and its mutant. Comparison of the C57 black and C3H agouti strains gave no indication of the pronounced taming effect of the black (aa) gene which Keeler reported in rats. With respect to aggressiveness toward other mice, the C3H showed more initial aggressiveness, but Ginsburg and Allee (1942) found the C57 blacks superior fighters over the C3H agouti and C albino strains, both of which carry the AA genes.

This technique remains as a possibility, but the ideal experiment is yet to be done. From general considerations pointed out above, it might be expected that any gene might have multiple effects, some of which might concern behavior. On the other hand, it would also be expected that most of these effects would be minor in nature.

It should be remembered at this point that all methods of study are dependent

upon raising the animals in a uniform environment, since all behavior traits may be largely affected by changes in the environment. This at once eliminates the pedigree method as a possibility, since the maternal environment in different families cannot be kept constant.

A second short method for studying heredity is to compare the behavior of animals from different genetic subpopulations under environmental conditions which are as identical as possible. If carefully done, this demonstrates that hereditary differences exist; and these differences may be used in experimental work for various reasons. However, in appraising the results, consideration must be given to the possibility of differences in maternal environment in the two populations which may be passed along from generation to generation, for such a survey may show up cultural as well as genetic heredity.

Scott (1942) made a survey of highly inbred mouse strains raised under the same conditions, studying aggressiveness as exhibited in the first fight. Since the animals were segregated before they began fighting, it was assumed that the chance of cultural heredity was very small. He found that under the conditions given, which involve frequent human handling, the C_3H strain was distinctly aggressive when matched against C albinos, whereas the C57 black strain (subline 10) was distinctly peaceful and that there was almost no overlap between the two strains. Later Ginsburg and Allee (1942) studied the same strains with respect to their ability to win fights (as distinct from the initiation of fights) and found that the C57 blacks developed into the best fighters, the C_3H next best, and the C albinos the poorest.

Later on, Scott (1944*a*) showed that the C57 blacks could be rendered very peaceful by appropriate handling procedures or extremely aggressive by experience in fighting; and it may be concluded that the kind of strain differences which appear is dependent upon the environment and training of the animals concerned. It may also be concluded that, where maximum variability of the environment is permitted, training is far more important as a cause of variability between these inbred strains than is genetics. It should be remembered, of course, that the differences observed are not so great as between wild and tame strains.

Hall (1934) selected two strains of rats for differences in what he called "emotionality" in an open-field situation, which was measured by the frequency of defecation and urination. The description of his experiments indicates that he did not use close inbreeding and that, as usual with most selection experiments, maximum differences were achieved at about 7 or 8 generations. He then tested the two groups of animals for differences in behavior in a fighting situation (Hall and Klein, 1942; Billingslea, 1941). The rats were graded for aggressiveness on a scale modified from that of Davis (1933), each rat being matched for 5 minutes with every other in a group of ten. Each group contained five rats of each strain. Hall and his associates found that there was a good correlation between what he called "aggressiveness" and between less timid behavior, as measured in the open field. These results are interesting but raise many questions. Hall does not seem to have paid any attention to dominance, and there seems to have been very little really active fighting in either strain. Much of what he called "aggressiveness" might also be considered simply fearless social investigation.

However, since the animals were kept isolated in cages, it may be presumed that there was no cultural effect present,

and it may be concluded that an important hereditary effect was demonstrated, whatever its precise nature. The correlation between fearlessness in the open field and aggressiveness is what might be expected if there is a consistent trait of aggressiveness running through many situations; but this correlation may, of course, be caused by accidental selection of two independent traits.

A more precise and difficult method of genetic analysis may be employed, using as a basis Wright's (1934) formula (originally modified from Castle) for the estimation of the number of genetic factors involved where variability is continuous:

$$N = \frac{\Delta^2}{8\,(\sigma^2_{F_2} - \sigma^2_P)}.$$

This formula and its variations are based on comparing the variance in the parental and various hybrid generations. If two homozygous strains are mated together, the F_1 individuals will be heterozygous but identical, so that any variability in the parental or F_1 generations must be due to the environment. Crossing the F_1's among themselves or making a backcross to one of the parental strains will increase the variability because of segregation, and the amount of increase produced will depend upon the number of genes involved. It will be noticed that, in order for the formula to work, there must be sufficient numbers in the parental strains so that an accurate estimation can be made of the average differences, and sufficient numbers must be raised in subsequent generations so that an accurate estimate of variance may be made. For a behavior trait the preferred experiment is one in which the F_1 is composed of approximately equal numbers from reciprocal crosses. This allows for the effects of maternal environment and does away with the problem of differential variability in the parental strains.

The limitations of the method are that it gives an estimate of the number of genes actually involved in the particular cross rather than the total possible number which can affect the character. Furthermore, if the parental stocks are only partially homozygous, results can be obtained only for the homozygous genes. Needless to say, strict control over environmental conditions must be maintained in successive generations.

Unfortunately, only one such experiment has been properly made, since most of the work of this sort was done before Wright's formula was developed. This particular experiment was that done by Dawson (1932), who measured wildness and tameness as measured by speed in a runway when the mice were urged from behind with a movable barrier. He used as parent-strains laboratory stocks of wild and mutant mice. These were probably somewhat inbred, but the amount was not measured. The fastest animals from the wild strains were mated with the slowest from the tame strains, and, through reciprocal crosses, F_1 and F_2 generations were produced. The results indicate that there were two or three genes involved and that the genes for wildness were dominant. There were no differences between reciprocal crosses, and in the wild strain the females appeared to be wilder than the males. By selection Dawson was able to show that the average of the tame strain could be altered, showing that these strains were not homozygous and that several more genes were actually present. In making the crosses, several tame strains were crossed with the wild, so that the results probably give a picture of the average number of genes which were homozygous in these stocks. This work definitely confirms the expectation of multiple factors listed in the beginning of this section.

Most of the other studies have been

made in such a way that no statistical analysis is possible, but they may be mentioned because of historical interest and because all of them tend to demonstrate that some hereditary effect was present.

The original experiment of this type was done by Yerkes (1913), and his results inspired others to attempt similar experiments. He crossed Castle's hooded rat with wild animals and graded them for savageness, wildness, and timidity in reaction to human beings. The results showed an F_1 which was extremely wild, savage, and timid, and demonstrated increased variability in the F_2, but with most animals near the timid grades. Most of the animals in the F_1 were grade 5 in savageness, whereas in the F_2 most of them were in grade 0. The results are peculiar from a hereditary point of view but indicate that possibly there was dominance of the wild traits in the F_1 together with hybrid vigor and that a multiple-factor ratio was apparent in the F_2. Since the strains were not pure and since small numbers were used in the parental strains, no conclusions can be drawn.

The next study was that of Utsurikawa (1917), who attempted to study the results of outbreeding and inbreeding, using Yerkes' tests as a model. Since he used an extremely small sample (14 animals in either strain) and made no crosses, no significance can be attached to the results, except that the material was very variable.

Stone (1932) made a cross between Wistar albinos (presumably inbred) and descendants of trapped wild rats. Only 10 animals were measured in the wild parental strain, which means that an accurate estimate of differences between the means cannot be made. The F_1 groups show a consistently greater variance than the backcross to the tame strain. The results can be attributed to the fact that the wild strain was not homozygous whereas the tame strain was inbred. Hence genetic analysis of the sort indicated above cannot be made.

A similar crossbreeding experiment by Coburn (1922) in mice may also be mentioned. Here both parent-strains were noninbred, and the result is that there are no significant differences in variability between the F_1's and the F_2's. Consequently, no conclusions regarding inheritance may be made, although the study has other interesting points.

The conclusion may be reached that a systematic study of the role of heredity in producing variability in agonistic behavior still needs to be made. Since such a study must inevitably be laborious, it probably should not be made without considerable preparatory work and should follow the following steps: (1) thorough study under various standard environmental situations of large numbers of two highly inbred strains known to be widely different; (2) development of tests of temperamental traits in several social situations, so that some conclusions can be reached as to whether we have a single underlying behavior mechanism which when modified will be part of all or many situations, or several underlying mechanisms adapted for particular situations and modified independently; (3) crossbreeding and measurement of animals in F_1 and F_2 or backcross generations; and (4) analysis of results both by Wright's method and by intercorrelation of traits, probably by factorial analysis.

In summary it may be pointed out that, while many of the data are not conclusive, all results are consistent with the hypothesis of traits affected by many hereditary factors with dominant genes producing the wild-type traits of savageness and wildness and with the expectation that environmental influences are very important. Final proof of multiple

effects on agonistic behavior by a single gene is still not available. Experiments on modification of aggressiveness and savageness described in a previous section support the expectation of the importance of environmental variability.

EXPERIMENTS WITH PHYSIOLOGICAL MECHANISMS OF AGONISTIC BEHAVIOR

Agonistic behavior, as well as other types, can be subdivided according to the results of training into two parts (Scott, 1947). The first of these, which may be called "nonmodifiable behavior' for want of a better term, is essentially reflex in nature. Learning affects it by as sociation only; and such a reaction may be conditioned either to appear or not to appear in response to certain stimuli, may be made stronger or weaker, but is never essentially changed in nature. Examples of such reactions in mice are tail-rattling and hair-fluffing, which appear externally, and the various accompanying internal reactions, such as changes in the heart rate.

On the other hand, the second type of behavior, which may be termed "modifiable," not only responds to learning of the simple association and inhibition type but is much more variable and adaptable to a wide variety of conditions. In this type of behavior, useless motions can be eliminated and the efficiency of the response improved. For example, such behavior as biting can be modified so that an experienced fighter may learn to disable his opponent within a few seconds by biting in the right place. Such modifications are not found in organs which are controlled entirely by the autonomic nervous system.

It has been noted that mice which have been trained to fight and have been uniformly successful almost never exhibit hair-fluffing and tail-rattling but at once attack violently. On the other hand, mice which have been defeated once and are presented with the opportunity of fighting exhibit these symptoms before starting to attack. The conclusion was drawn that these reactions are part of a mechanism which comes into effect when the animal is equally stimulated to escape and attack and that other mice might learn to interpret this behavior as a warning.

These results apply to one part of the important problem of what happens within the body after environmental conditions are such that conflict behavior follows. The nonmodifiable behavior referred to above largely includes the sorts of behavior which are loosely termed "emotional." It is suggested that the process of positive and negative learning in male mice makes possible situations in which an animal can make no adaptive reactions. As a protection against this possibility there apparently exists a mechanism whereby nonmodifiable behavior connected with conflict behavior may do at least two things: (*a*) produce external reactions which may act as signals to other animals and (*b*) in extreme cases may possibly take control over organs normally used in modifiable behavior.

The latter possibility is not one which has been observed in mice or rats but is one which stems from Masserman's (1943) experiment of directly stimulating the hypothalamus of cats, which "lost control" over themselves to the extent of striking and scratching, actions which are usually under "voluntary" control and belong to the class of modifiable behavior. The experiments of Cannon (1929) and Masserman (1943) point to the existence of a center of nervous control working through the hypothalamus and, in turn, chiefly through the autonomic nervous system, the adrenal me-

dulla, and various motor nerves which are normally "voluntary." This center has two functions in addition to those listed above: (*a*) increasing the efficiency of physiological reactions by altering the rate of breathing, heart rate, etc., and (*b*) providing sensations which may act as additional stimuli for modifiable behavior, particularly if learning is involved. These results have not been confirmed on mice and rats, but the anatomical and physiological characteristics of mammalian species are similar enough to warrant the tentative extension of these findings.

The work of Selye (1946) on the rat points to the existence of a second center of control in the hypophysis, which may or may not be linked with the above and which operates largely through the hormones of the adrenal cortex, which, in turn, have profound effects on metabolism, the circulatory system, and the kidneys.

This center of control reacts in some way to any injury to the body (probably in reaction to substances from injured tissues) and also to fear and rage. In a defeated mouse which has been injured by its opponent, both sorts of reactions would be expected to be present. From the nature of its effects, it is obvious that the center can affect conflict behavior chiefly in two ways, by altering the efficiency of physiological processes and by producing sensations which act as nervous stimuli.[5]

Selye has described the results of action by this center as a syndrome; and, during the initial or shock phase following stimulation, body efficiency is lowered by tachycardia, decrease in body temperature and muscular tone, decrease in blood sugar, etc. These symptoms are reversed and body efficiency increased in the phase of countershock, which may follow as soon as a few minutes later. This may account for the fact that trained fighters which are "excited" before a fight are much more efficient than those which are not.

It may be postulated that these reactions of shock and countershock should also cause sensations within the body which may contribute to the type of behavior which results. The later phases of "resistance" and "exhaustion" probably affect behavior only in the case of long-continued and habitual fighting but may have some relationship to recovery by long-defeated mice.

Before further conclusions can be made specific, experiments need to be done on the relationship between the "general adaptive syndrome" of Selye and agonistic behavior. Do all or only part of the components of the syndrome appear in connection with fighting? What are the physiological states of a winning fighter, his defeated subordinate, or a repeated loser? These and many other questions are still to be answered.

DISCUSSION AND CONCLUSIONS

A. THE ADAPTIVE NATURE OF FIGHTING BEHAVIOR

One general problem mentioned in the introduction is that of the adaptive nature of social or intraspecific fighting in mice and rats, as distinguished from interspecific fighting, which may be assumed to be useful in predator-prey relationships. Considered from the point of view of the individual, there is occasionally some advantage in competitive fighting which enables the winner to get a piece of food. However, the most important type of social fighting in mice and

[5] The existence of this new evidence may explain some of the discrepancies in early experiments, as, for example, the fact that the injection of adrenalin does not produce sensations of anger and that sympathectomized animals may still appear to be emotionally aroused in conflict situations.

rats appears to be noncompetitive in nature, and its existence may therefore be the product of the artificial conditions under which those animals are raised, or it may be an injurious trait which has accidentally arisen in evolution, or it may be a trait which has some use for the species as a whole (Allee, 1942*b*). The latter appears to be the most likely hypothesis; for, wherever rats and mice live in g oups, some social organization results and fighting may be of use to the society as a whole, even though it may appear to be merely destructive for an individual.

Calhoun (1949) has found with his studies of rats under seminatural conditions that fighting behavior appears to result in controlled use of living space. His rats had unlimited supplies of food and water, but, whereas caged rats can live with only 2 square feet of living space per individual, his population leveled off at 50 square feet per individual. It may be supposed that, if his rats had been able to escape, many of those individuals would have emigrated from the area and that the result of fighting in these animals is to maintain a rather fluid and expandable type of social organization, which is always ready to make use of any vacant living space but which will not permit more than a certain maximum density of population, even with an unlimited food supply.

Scott has suggested that fighting in rats and mice may also tend to prevent homosexual activity and to insure distribution of the sexes through the living space, so that fertile mating behavior is easily achieved. The fact that female mice are less likely to fight and are easily dominated by the males makes it difficult for them to reject the advances of males. It is therefore possible to assume that part of the mechanism of fighting within the species is primarily concerned with insuring fertile matings.

Calhoun found that where male rats were not organized into a stable dominance order, groups of males would follow a female in estrus and repeatedly and briefly copulate. Such females showed a relatively low rate of conception (possibly because no single male was ever able to reach a state of ejaculation). If this observation is correct, fighting and dominance would lead to a more favorable condition for reproduction among rats.

On the negative side it may be stated that competition over food and sex does not seem to be an important cause of fighting. Competition over females does not seem to occur, and competition for food does not appear except in special experimental situations. As Calhoun has pointed out, the so-called "hoarding" (really, food scattering) of rats actually tends to make more food available for the whole population, since these deposits are not guarded by the individuals who make them.

Further studies of the natural social organization of mice are needed before the role of social fighting can be evaluated, as has been done with Calhoun's rat studies.

In the case of rats, and to a lesser extent in mice, fighting behavior is undoubtedly of use in fighting off predators and capturing small animals for prey. The conclusion of Collias (1944) that a decrease of fighting outside a group is accompanied by an increase within a group suggests that fighting within the species might be solely a misuse of behavior which is otherwise adaptive, and dominance might be considered as merely a primitive method of controlling the undesirable aspects of a necessary behavior trait. While this is a possibility which must be kept in mind, the existence of fighting behavior within the species will alter social organization, whether for use

or for harm. Further, if interspecific fighting alone were useful, there would be no explanation for differing fighting tendencies in the two sexes.

In summary of these various points, it may be said that interspecific conflict behavior appears to be adaptive in predator-prey relationships. Conflict behavior within the species (social fighting) appears to have two uses: to produce an expanding and fluid type of society without definite territories, which makes for the optimum utilization of living space, and to insure fertile sexual behavior. There has apparently been selection toward lessened aggressiveness on the part of females, so that fighting will not interfere with male-female sexual behavior. Dominance, or the social relationship springing out of habitual conflict behavior, has the function of regularizing space utilization and sexual relationships, as well as minimizing the harmful and destructive aspects of social fighting.

B. RELATIVE USEFULNESS OF THE TWO SPECIES FOR EXPERIMENTAL ANALYSIS OF CONFLICT BEHAVIOR

Enough work has been done upon the conflict behavior of the two species so that it is possible to evaluate them in terms of usefulness for laboratory investigation on this subject. It must be concluded that mice are probably better than rats for laboratory experiments on fighting. Most of the domestic strains are more aggressive than the rats, and they are much easier to raise and control. However, because of their larger size, rats are probably better suited than mice for field experiments and for physiological analysis of fighting behavior.

It may be pointed out that both species have definite limitations with regard to certain aspects of fighting. Because these animals exhibit relatively little allelomimetic behavior (two or more animals doing the same thing at the same time, often involving mutual imitation) compared to animals like sheep or dogs (Scott, 1950), group attacks upon an individual or fighting between groups cannot be studied. It is not possible to study sibling rivalry or sexual jealousy as causes of fighting, since these factors appear to be absent or negligible. And, because of the relatively small size of both species, there are limits to what can conveniently be done with physiological analysis of fighting.

On the positive side it should be pointed out that their relatively small size makes both species excellent for neurophysiological experiments. Smallness makes operations difficult, but it makes subsequent microscopic examination of tissues much easier.

The two species are likewise ideal for experiments with training and heredity. Inbred stocks are available in both species, and there is a particularly wide variety in mice. As a glance at the extensive psychological literature on the subject will show, rats and probably mice are capable of associative learning and can solve problems of considerable complexity.

As has been pointed out above, considerable progress has been made along the lines of analyzing the effects of variability in heredity and variability in training; and, if the above limitations are kept in mind, it may be considered that a fairly sound (though by no means complete) foundation has been laid for the study of abnormal behavior which may arise in situations of conflict in the two species. Furthermore, these studies may be considered a guide to the extension of findings to other species, so that eventually we may have the basic material for sound generalizations regarding fighting behavior.

C. THE PHYSIOLOGICAL MECHANISMS UNDERLYING CONFLICT BEHAVIOR

We may now summarize the basic mechanisms which appear to lie behind fighting behavior in the two species, considering first the mechanism involved in reacting to an immediate situation and then the mechanism through which previous conditions affect behavior (see Fig. 1). For simplicity, the discussion will be limited to a noncompetitive situation.

Fighting behavior appears to start with external stimuli or changes under specific conditions. These involve the presence of a strange animal, the pain of an attack, or the sight of another animal running away. These stimuli affect the cutaneous nerve endings and the mechanism of sight[6] and then are carried to the cerebral cortex and probably to some sort of lower controlling center (it should be emphasized that these neural centers and much of the internal mechanisms are hypothetical and derived from knowledge of other species). Nerve impulses from the cerebral cortex result in the modification of the voluntary part of fighting, such as scratching, running, and biting.

Impulses from the lower center may have many functions, all affecting nonmodifiable behavior. Some of these act through the hypothalamus to set up adaptive responses in the heart, adrenal glands, and other parts of the body; to produce external signaling behavior, such as tail-rattling and hair-fluffing; and under extreme conditions to cause automatic biting and scratching of the reflex nature. Sensations of the physiological changes in the body may be carried to the cerebral cortex and set up associations with fighting and probably give rise to new impulses.

[6] Sight is not necessary for fighting to take place, according to Beeman.

Impulses from the same or perhaps another center activate the anterior pituitary, which, in turn, sends hormones to the adrenal cortex. Hormones released from the area modify the action of the circulatory system and produce changes in metabolism which result eventually in increased physiological efficiency for dealing with an emergency and probably also in sensations which are returned to the cerebral cortex and may set up new impulses there.

Whether or not these activities are controlled by the same center which controls those mediated through the hypothalamus and autonomic system is not known. Whether either group of reactions is unitary is likewise a matter of doubt.

This basic mechanism may be modified by other external factors. Limited food supplies may cause two animals to compete, leading to pain and thus initiating the mechanism of fighting. Physical factors such as heat may set up competing adaptive reactions which tend to slow down or reduce severity of fighting. Variations in space may cause two animals to choose different reactions which are adaptive to the situation.

Hypothetical as this schematic system is in many respects, one very important point stands out: There is no evidence for any sort of spontaneous internally arising stimulation which would cause a "need for fighting" per se. Instead, we have a mechanism which will produce fighting in response to specific and predictable external stimulation. Even the male hormone apparently produces differences in response levels between individuals rather than creating stimulation within an individual (see Fig. 2).

This conclusion apparently conflicts with the possibility stated by one of the authors in an earlier paper (Fredericson, 1946), that "aggressiveness stems from a

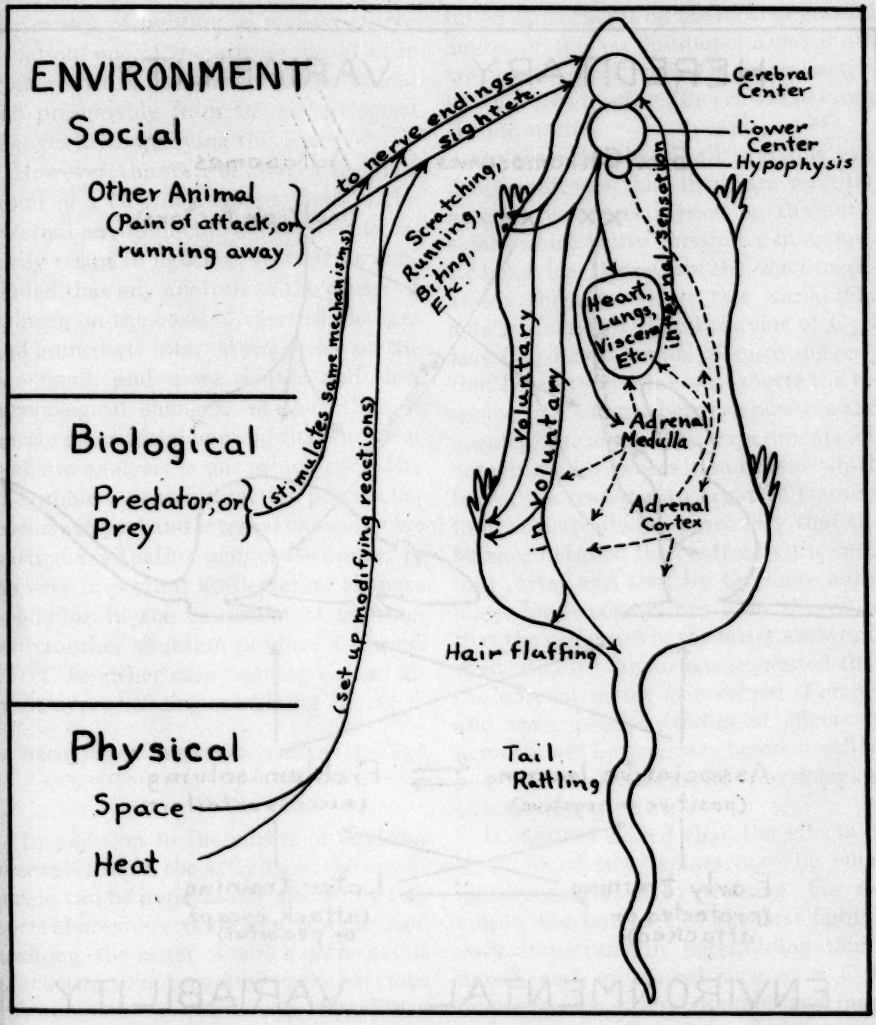

FIG. 1.—Mechanisms affecting the origin of fighting behavior, particularly of the noncompetitive type. Nervous stimulation is shown in solid lines, hormonal influences in dotted lines. All details are not shown because of lack of space; but it is evident that there is no place in the diagram where spontaneous internal activity might arise to create a need for fighting per se.

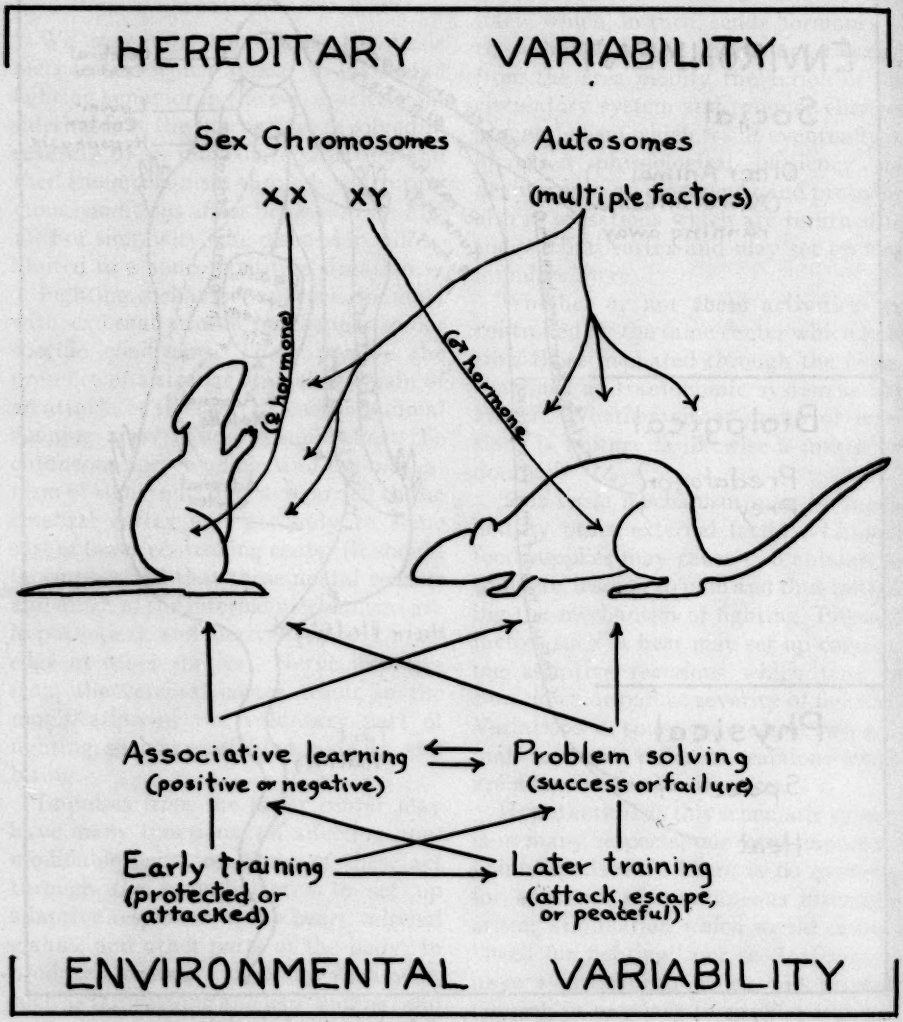

FIG. 2.—Mechanisms affecting variability and consistency in agonistic behavior. This diagram shows the major sources of variability in the behavior of animals tested under similar conditions.

basic energy source in the organism." Indeed, if a similar diagram were made of the causes of fighting in a competitive situation, one of the arrows would arise from a state of hunger within the animal and presumably from the physiological changes accompanying this state.

However, the state of hunger itself is a result of a lack of available food in the external environment, nor does it necessarily result in fighting. It must be concluded that any analysis of the causes of fighting on the basis of external changes and immediate internal responses, on the one hand, and more remote and slow physiological changes, on the other, is purely an artificial one and that the most realistic analysis is one which considers the problem as an interaction process between external and internal changes. One particular situation may cause hunger to be very important and external changes negligible in the causation of fighting, and another situation produce a reverse effect. In either case training has an almost overwhelming modifying effect.

D. MECHANISMS AFFECTING VARIABILITY AND CONSISTENCY IN CONFLICT BEHAVIOR (FIG. 2)

In addition to the effects of environmental stimuli, the activity of the mechanism can be importantly altered by two sorts of previous conditions: heredity and training, the latter of which is the result of reacting or not reacting to the previous environment. Considering hereditary variability first, the most precise mechanism known is that involving sex. Factors on the sex chromosomes cause a differentiation between male and female hormones which may produce an enormous difference in the degree of response to environmental stimulation. Animals possessing the male hormone are, of course, very easy to stimulate. How the male hormone produces this effect is still unknown, and the field is open for detailed experiments on the local or general action of the sex hormone. Does it increase pain sensitivity? Or general activity? Or does it affect the central nervous system itself?

On the other hand, all available evidence indicates that there are multiple hereditary factors carried on the autosomes which cause variability in agonistic behavior. Here again the exact mechanism through which this variability acts is unknown. The behavior of C3H and C57 black strains of mice suggests that heredity in some way affects the response to a balance between positive and negative training. The experiments on hereditary differences in tameness, which is really a response to negative training to fight, indicate the possibility that the balance between the control by the cerebral cortex and that by the more automatic lower centers has been altered so that the responses of the latter are weakened. Richter (1950) has suggested that the adrenal cortex is involved. Further and more precise studies of effects of heredity will be necessary before it will be possible to evaluate its physiological effects more precisely.

It may be added that the effects of small hereditary factors may be enormously magnified by training. For example, the outcome of the first fight is very important in determining dominance, and an animal such as a C3H mouse, which is less vigorous and more hesitant, may well lose this first fight to a C57 black and continue to lose on subsequent occasions, even though the initial behavior differences were relatively slight.

Regarding the effects of training itself, there are at least two types of learning: that involving simple association (positive or negative learning) and that in-

volving the solution of problems. Simple associative learning can affect both activities under the control of the lower centers and those under the control of the cerebral cortex; and, as the studies of Ginsburg and Allee (1942) and Seward (1945) have brilliantly established, the same principles apply here as those which Pavlov derived from his study on the salivary reflex. Theirs was the first demonstration that the laws of associative learning apply to a major basic type of social behavior, as was anticipated by Miller and Dollard in their study of imitation (1941), and may be considered as a model for extending this principle to other types of social behavior. Associative learning can cause an animal to be consistently aggressive or consistently timid and fearful. This is one of the most noticeable effects of training, for inexperienced mice facing a conflict situation for the first time show extremely variable and unpredictable behavior.

The process of problem-solving accounts for this preliminary variability, and this appears to be its most important effect. The animal simply tries out in succession the various simple adaptive patterns of behavior which are peculiar to the species. No great complexity of behavior is involved, and associative learning or habit formation fixes the reaction which gives most successful adjustment.

The evidence cited in this paper points overwhelmingly to the conclusion that training includes by far the most important group of factors which affect agonistic behavior. Passive inhibition causes mice raised together to be peaceful instead of combative; and previous success or defeat makes the difference between a mouse which will attack all comers and one which runs away from any other animal. The effects of primary stimuli or releasers can be overcome by training, which also withstands the effects of almost any physiological change short of death. The greatest known differences produced by heredity can be duplicated or exceeded by differential training applied to a single genetic type of animal. Important as other factors may be under special situations, it must be concluded that the most important cause and hence the most important factor of control of fighting and agonistic behavior in rats and mice is training, which includes the interaction of the two processes of associative learning and problem-solving.

SUMMARY

1. Fighting may be considered one of a group of related behavior patterns which may be termed "agonistic."

2. This paper is an attempt to organize the available data concerning the factors which affect agonistic behavior in two fairly closely related species, mice and rats.

3. Agonistic behavior patterns are described in detail, and a brief outline of important experimental methods is given.

4. In "noncompetitive" situations (i.e., not involving fighting over objects), developmental study indicates that the male hormone and pain are important factors affecting the initial appearance of fights. Pain, noise, sudden movements, and restraint have been used to produce agonistic behavior in response to direct human stimulation.

5. In competitive situations the important causal factors appear to be states of hunger and possibly thirst and the degree to which fighting is an effective response in a given situation. Thus frustration tends to give aggression only in situations in which aggression provides a satisfactory solution to a problem.

6. Experiments with experienced or trained animals have been largely done with animals which have developed a dominance order when fought by the

round-robin technique. Cold has no effect within the temperature ranges tested; heat appears to inhibit aggressiveness. Increased space modifies the patterns of behavior and permits a vague sort of territorialism.

7. Where dominance is already established, competition among hungry animals for food results in cutting down the usual amount of fighting. It is concluded that *frustration as a cause of aggression is important only when animals have been trained to react aggressively after frustration.*

8. Avitaminosis and other nutritional deficiencies may reduce the amount of fighting of a dominant animal but have little effect on dominance, short of death.

9. Male mice trained by the round-robin technique and then castrated continue to fight, indicating that, once the habit of fighting is established, the complete supply of male hormone is not necessary to maintain fighting (some androgenic hormone may be furnished by the adrenal glands).

10. Both males and females will compete for food, indicating that the male hormone is of little significance in this situation. Hunger is not necessary to produce fighting over food, once the habit has been established.

11. Training in early life produces important effects on fighting tendencies in later life, whether or not object-competition is involved.

12. The principles of learning are described; experimental data concerning dominance confirms their application to fighting behavior in both mice and rats.

13. Based on these principles, methods are described for training mice to fight, run away, or be peaceful.

14. It would be theoretically expected that heredity would affect fighting behavior through multiple factors; that factors for "wildness" and "savageness" would be dominant in the first generation; that pleiotropic and interaction effects would be observed; and that in most situations environmentally controlled variability would exceed genetic variability.

15. Methods for the genetic study of behavior are described, and it is concluded that an ideal experiment should include, among other things, a wide variety of tests covering various situations.

16. All results so far obtained agree with the expectation in paragraph 14. In addition, the kinds of genetic difference obtained vary considerably with the nature of the test situation, indicating that the traits involved may be very specific.

17. Two controlling centers of physiological reactions connected with agonistic behavior appear to exist—a nervous center in the hypothalamus and an endocrine center in the pituitary body.

18. Fighting behavior which is not object-centered has two adaptive effects: to regulate living space and to insure fertile sexual behavior. The adaptive nature of object-directed fighting is obvious. Dominance has the effect of regularizing space utilization and sexual relationships, as well as minimizing the harmful and destructive aspects of social fighting.

19. The relative usefulness of rats and mice for research on fighting is discussed.

20. The network of causal relationships through which fighting is produced is summarized. In some situations external changes are very important; in others internal changes may be very important. No center of internal change has been discovered which might create a "need" for fighting per se.

21. It is concluded that training includes by far the most important group of factors which affect consistency in agonistic behavior, although hereditary factors (and particularly the male hormone) may be very important in certain

situations and various environmental and physiological factors may be important in determining whether or not fighting is started in the first place.

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FACTORS INFLUENCING THE STATE OF DISPERSION OF THE DERMAL MELANOPHORES IN RAINBOW TROUT*

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IT IS a common observation that trout not only vary in color according to the light intensity of their home environment but also become paler or darker as they move to backgrounds of greater or lesser illumination. However, the rate at which such changes in shade occur has apparently received little study, notwithstanding the interesting implications, both ecological and physiological. During the course of previous investigations on the transformation in color of rainbow trout (Robertson 1948, 1949) incidental observations revealed the fact that these fish exhibit a wide variety of responses to different stimuli with respect to rate and degree of color change. Further study soon indicated that adequate interpretation of such melanophore reactions, especially those of isolated skin strips, would necessitate the acquisition of much more detailed knowledge of the conditions affecting the state of dispersion of these cells in the trout. Although the literature contains extensive investigations on the effect of different environmental conditions and chemical compounds on the activity of a variety of teleost melanophores (reviewed by Parker,

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1948), few studies on trout are to be found. Moreover, most previous workers have focused attention on some particular factor or phase of melanophore activity; hence their findings, while illuminating various aspects of the field, provide only a partial description of the conditions which control the functioning of these pigment cells in any one species.

The dermal melanophores of the rainbow trout (*Salmo gairdnerii*) lend themselves particularly well to systematic study. They react quickly, are very sensitive to certain changes in environment; they can be readily isolated from the body by stripping off the skin and are of sufficient size for satisfactory examination. The present communication consists of observations on the effects of various factors—physiological, physical, mechanical, chemical, pharmacological, and hormonal—which have been found to influence the state of the dermal melanophores in this species of trout. Less extensive studies have been made in the brown trout (*S. trutta*) and the brook trout (*Salvelinus fontinalis*).

MATERIALS AND METHODS

TROUT

The trout employed were one to two years of age, 6–8 inches long, both hatchery-reared[1] and wild. The latter were caught on artificial flies shortly before use. The fish were kept in relatively dark-colored troughs containing an adequate flow of water at 50° F. The pH of the water was 7.8 and the O_2 content 7–8 p.p.m. A complete description of the environmental conditions is given in a former publication (Robertson, 1949).

OBSERVATIONS ON INTACT FISH

The changes in color or shade which occurred in the living fish under varying experimental conditions were usually sufficiently distinct to leave no doubt, but, when such existed, examination was made under the dissecting microscope. Fish were killed by a sharp blow on the head or by severing the spinal cord near the head. Under such conditions no change in the state of the dermal melanophores occurred for a considerable period of time.

PREPARATION OF SKIN STRIPS

Strips of skin, approximately 10 mm. wide and extending from near the mid-line of the back to the belly, were easily peeled off from the subcutaneous tissues and placed directly in the test solutions. Control strips were left in 0.9 per cent NaCl solution or a balanced solution containing NaCl 0.8 per cent and KCl 0.1 per cent with pH 7.6–7.8 and kept at a temperature of 10° C. Also in the latter part of the work a drop of the test solution was placed on the subcutaneous surface of a skin strip laid epidermal side down on filter paper moistened with NaCl solution and contained in small Petri dishes kept cold. This latter procedure makes it possible to study certain reactions in the absence of ambient fluid. Examination was made under the dissecting microscope. Permanent mounts of these preparations were carried out as described in an earlier publication (Robertson, 1948).

For the sake of brevity, the terms "dispersion" and "concentration" of melanophores are employed to indicate the dispersed and concentrated states of the pigment granules within these cells. When concentration of solutes is discussed, the term "contraction" is used to designate the concentrated condition of the melanophores.

MORPHOLOGY AND DISPOSITION OF MELANOPHORES IN THE SKIN

The melanophores of the rainbow trout's integument are confined to the dermis and subcutaneous tissues. They appear in places to penetrate the epidermis, but in this location they are attached to the caudal (external) part of the scale. The dermal melanophores in their dispersed state, which seems to be the normal one for the trout, exhibit many fine branches with tapered ends (Pl. I, Fig. 1). Under the various influences which produce pallor of the trout, the degree of

[1] The author wishes to express his appreciation to Mr. James Scully, regional supervisor, Michigan State Department of Conservation, Marquette, Michigan, and to Mr. Martin Miller, superintendent, States Fish Hatchery, Thompson, Michigan, for the experimental trout used in this study.

concentration varies considerably from cell to cell (Pl. I, Fig. 2). The last to show concentration are those attached to the scales, which, under even very potent melanophore-concentrating influences, may still remain in a state of partial dispersion.

Melanophores of the subcutaneous tissues differ markedly from those of the dermis both in morphology and in reactivity. They are of two kinds—discrete-appearing cells with rather short blunt branches shown in Plate I, Figure 3, and a syncytium-like structure which characterizes the melanophores of the parr marks. That these latter cells are actually individual units was shown by the injection of a relatively strong preparation of adrenalin into a parr mark, which caused concentration into discrete units (Pl. I, Fig. 4). These subcutaneous melanophores appear not to be affected by various influences which cause marked changes in the state of the pigment cells in the dermis. When they are made to respond by artificial means, e.g., locally introducing high concentrations of melanophore-contracting substances, their response is slow in comparison with the dermal pigment cells.

PHYSIOLOGICAL FACTORS

ASPHYXIA

It is well known that asphyxia produces pallor in the trout, but, in studying this process, it was of interest to find that the oxygen lack had to reach an extreme stage before concentration of the melanophores occurred. A trout taken out of water survived for 8 minutes and showed no pallor for the first 5 minutes, in spite of violent convulsive movements and marked dyspnea. After this time the onset of pallor was gradual and progressive until death. Another trout kept in about 500 cc. of cold water remained alive for 5 hours. Although dyspnea began after the first hour and a quarter and increased steadily, concentration of the melanophores was not noted until 10 minutes before death.

Deep ether anesthesia likewise produces concentration of melanophores and pallor, whereas no change occurs with anesthesia just sufficient to immobilize the fish. These asphyxial effects are readily reversible if the trout survives.

Instant death, as mentioned earlier, prevented subsequent concentration of the melanophores. However, if this was not achieved and the fish gasped a few times before expiring, concentration of the pigment cells soon began to occur.

That the asphyxial effect is a central one was shown by cutting the nerves supplying a small area (1.5–2 cm. in diameter) of the skin of the back just anterior to the dorsal fin. Such an area failed to blanch with O_2 lack due to taking the fish out of water or with deep anesthesia. These tests were carried out 24 hours after cutting the nerves and again 10 days later, which allowed time for complete nerve degeneration and re-establishment of the circulation, which may have suffered damage from the incision.

To determine whether or not the effect of asphyxia was due to increased CO_2 in the blood, oxygen containing 10 per cent carbon dioxide was bubbled through water contained in a bucket painted black inside. Trout in such water showed pallor beginning within 3–4 minutes, became very distressed, and exhibited convulsive movements. It was found that the pH of this water had changed rapidly from 7.8 before to 5.8 after bubbling the O_2-CO_2 mixture through it. Essentially the same effects, though somewhat less pronounced, were produced when trout were placed in well-oxygenated water which had been acidified to a pH of 5.8 by means of N/$_1$HCl. For obvious reasons it was not possible to

separate the effects of increased CO_2 content of the water and increased hydrogen-ion concentration. Contraction of the melanophores produced by increased CO_2 and acidity of the water was shown to be of central origin, since the denervated skin failed to blanch under these conditions. Furthermore, the isolated skin strip placed in 0.9 per cent NaCl through which the O_2-CO_2 mixture was passed did not exhibit concentration of melanophores, even though the pH of the solution fell to 5.2.

teleosts, including trout (Parker, 1943, 1948), appears to depend on the illumination intensity of the background. The rate of color change exhibited by rainbow trout on changing from black to white backgrounds and vice versa is among the most rapid that has been recorded for melanophore response. The data shown in Table 1 were obtained by placing the fish alternately in white and black buckets exposed to full sunlight. Prolonged exposure—hours to days—on a white background produced a somewhat increased pallor. The most conspicuous change, however, occurred within the time intervals shown in Table 1 and involved the melanophores of the back, sides, and ventral surface. Trout kept for 60 hours in a white bucket,[2] then placed on a black background, showed a return to the normal dark color of the back and sides within 3–4 minutes but the ventral surface remained pale for more than 24 hours. However, when such a fish was killed, the belly melanophores soon re-expanded.

TABLE 1

RATE OF COLOR CHANGE PRODUCED IN RAINBOW TROUT BY TRANSFERRING TO LIGHT AND DARK BACKGROUNDS

Dark to Light	Time Interval	Light to Dark	Time Interval
Beginning pallor	45 sec. to 1 min.	Beginning darkening	30 sec. to 1 min.
Marked pallor	1½ min.	Marked darkening	1–11 min.
Maximum pallor	2 min.	Maximum darkening	2–3 min.
Repeated Tests			
Beginning pallor	30 sec.	Beginning darkening	30 sec.
Marked pallor	1 min.	Marked darkening	1 min.
Maximum pallor	1¾ min.	Maximum darkening	1–2 min.

Absence of oxygen did not interfere with the rate or degree of concentration of melanophores in the isolated skin strip exposed to dilute solution of adrenalin.

OTHER FACTORS

Trout starved for a period of 7 weeks failed to exhibit any change in color. During this period a loss of from 7 to 9 per cent in weight occurred. No evidence of melanophore concentration was observed as a result of excitement due to netting, handling, needle puncture, or cutting skin and muscle, even though the fish struggled violently.

PHYSICAL FACTORS

LIGHT

The effect of light, as has been found by numerous other writers on different

Observations on the role played by the eye in mediating the color changes resulting from variations of background light intensity corroborated those of earlier investigators on other teleosts

[2] A sufficient fresh-water flow was maintained to keep the trout in good condition.

(Frisch, 1911). A trout under light ether anesthesia placed on a white background did not show pallor until it recovered from the effects of the anesthesia. A blind fish (bilateral cataracts) showed no pallor on changing to a white background. Killing a fish on a white background resulted in a rapid return of dark coloration. Section of one optic nerve produced contralateral darkening, an observation first made by Pouchet in 1877. This effect was brought out most sharply by placing the fish on a white background. Severing the nerves to a local area of the skin of the back abolished the melanophore-contracting effect of a white background. Light had no detectable effect on the melanophores of the intact dead trout or those in isolated skin strips.

TEMPERATURE

Raising the temperature of the water produced no alteration in the state of the melanophores of the trout until a lethal level was reached at 25° C. Then concentration occurred but only shortly before death.[3] On the other hand, melanophores of the dead fish kept in water or those of isolated skin strips in 0.9 per cent NaCl solution exhibited progressive shortening of the time of beginning concentration as the temperature was raised from 10° to 25° C. These findings, representing the average results of a number of experiments at each temperature, are presented in Table 2. They agree in general with Smith's (1928) conclusions concerning the effect of temperature on the isolated melanophores of *Fundulus heteroclitus*. How long these cells would remain in the expanded state in solutions at temperatures of 1°–5° C. was not determined, but in one experiment they had not changed at 8 hours. Skin strips on moistened filter papers kept at 4° C. for 24 hours showed dispersed melanophores, which concentrated when adrenalin was applied. Since the melanophores remained in a stable state of dispersion in 0.9 per cent NaCl at 10° C. for more than 2 hours, both in the dead fish and in skin strips, and since this was the temperature of the water in which the experimental fish were maintained, all experiments testing influences other than temperature were conducted at 10° C.

[3] In the experiments conducted at 20° and 25 °C., air was bubbled continuously through the water.

MECHANICAL FACTORS

TRAUMA

Injury produced by incision through the skin of the live or dead fish[4] or maceration of the melanophores of isolated skin strips appeared to produce no effect on their state of dispersion.

TABLE 2

EFFECT OF TEMPERATURE ON MELANOPHORES OF ISOLATED SKIN STRIPS

Temperature of Solution (° C.)	Concentration of Melanophores Beginning to
3	8 hr.
5	8 hr.
10	2–4 hr.
15	50 min.–2 hr.
20	30 min.–1 hr.
25	15–30 min.
30	15 min.

PRESSURE

On the other hand, pressure, even of relatively slight degree, produced a pronounced concentrating effect both in live and in dead trout but not in the isolated skin strip. Demonstration of this effect in the live fish (described by Frisch, 1911) was made by placing a rubber band

[4] Dead fish placed in 0.9 per cent NaCl. In water, concentration occurred along the margin of the cut, owing to the action of the water; and, if in air, a melanophore-concentrating substance liberated from the muscle (described later) produced the same effect.

firmly but not too tightly around the body just anterior to the dorsal fin. Removal of the rubber after 40–45 minutes revealed a band of well-marked pallor encircling the body. This began to fade within a few minutes and disappeared at the end of an hour. That this effect was not mediated by nerves was demonstrated by employing a fish in which the nerve supply to a localized area of skin had been severed. Pressure of the rubber band produced just as marked pallor on this area as on the adjacent skin with intact nerves.

Pallor occurring in areas of pressure on the dead trout is a well-recognized phenomenon. At room temperature the underside of a fish laid on a hard surface will become pale within half an hour or less. If the fish is turned over, the concentrated melanophores will gradually disperse, while those of the underside will now show concentration. This reversal phenomenon, dark to pale and pale to dark, could be induced repeatedly. The same sequence of events occurred when the fish was laid on the glass stage of the dissecting microscope, showing that light did not play a role in the reaction. Low environmental temperatures were found to retard these changes and inhibit them completely at 0° C., as shown by laying a fish on a block of ice. The underside in contact with the ice remained just as dark as the upper, but at the end of about an hour both sides exhibited a transient pallor, following which the whole trout remained dark for the 4-hour period of observation. This same phenomenon of transient pallor—lasting a few minutes—has been observed in dead trout kept in water at 3°–5° C.

Duration of pressure rather than intensity appears to be the important factor in causing concentration of the melanophores. The very slight pressure of a leaf or one of the fishes' own fins in contact with the body will produce a sharply defined area of pallor.

The failure of melanophores in the isolated skin strip to respond to pressure or, at most, only to a very slight degree indicated the probability that connection with the underlying tissues was necessary for this effect. Such was found to be the case. Pressure on excised blocks of tissue consisting of skin and underlying muscle produced concentration of the dermal melanophores just as if the tissue were still a part of the dead fish. This finding suggested that pressure brought about a transfer of some melanophore-concentrating substance from the muscle or subcutaneous tissues into the skin.

Further investigation revealed that the muscle contained a potent concentrating substance demonstrable by contact of excised skin strips with exposed muscle of the living or dead trout or with saline extracts of muscle. Chemical analysis of such muscle extracts showed a high potassium content.[5] Since the potassium ion, as will be shown in the next section, produces marked concentration of melanophores and since appropriate tests excluded the action of organic compounds, it seems probable that potassium constitutes the active principle of this phenomenon. A more complete account of this phase of the study will be published elsewhere.

CHEMICAL INFLUENCES

IMMERSION IN VARIOUS FLUIDS

Isolated skin strips placed in water at 10° C. began to show concentration of melanophores within 30 minutes. The fact that the intact dead trout kept in cold water remains dark for a number of

[5] The author is much indebted to Dr. Lillian Eichelburger, department of surgery, University of Chicago, for determining the potassium content of the trout muscle and muscle extracts.

hours shows that water does not readily penetrate the skin.

The effect of varying percentages of NaCl solution is shown in Table 3. The melanophores of skin strips suspended in 0.5–0.9 per cent salt solutions remained in their dispersed state. At 0.4 per cent and below, concentration occurred. In 1.0–3.0 per cent NaCl solutions, well-marked dispersion took place. This latter effect was demonstrated by using skin strips previously blanched in water.

Potassium chloride caused concentration of the pigment cells in concentrations as small as 0.01 per cent. To differentiate between the melanophore-concentrating action of the potassium ion and that of water, drops of the test solutions were placed on the subcutaneous surface of skin strips. Under these conditions water had no effect, owing presumably to lack of penetration into the subcutaneous tissue, while dilute KCl solutions produced marked and rapid local concentration. When skin strips are immersed in water, the melanophore concentration beginning at the cut edges of the strip suggests that water reaches the pigment cells by this route. A drop of 0.2 per cent NaCl on the undersurface of the skin likewise failed to cause concentration, which indicates that the Na ion does not aid the penetration of water.

A balanced NaCl-KCl solution was found to consist of NaCl 0.8 per cent and KCl 0.1 per cent (Table 3). $CaCl_2$ also produces concentration of the dermal melanophores, but its action is much weaker than that of KCl. A concentration of 0.8 per cent NaCl will counteract the concentrating effect of 0.6 per cent $CaCl_2$.

TABLE 3

EFFECT OF VARYING PER CENT SOLUTIONS OF NaCl AND KCl ON MELANOPHORES OF ISOLATED SKIN STRIPS

A. *NaCl:*					
Per cent NaCl	0.1–0.2	0.3–0.4	0.5–0.9	1.0	2.0–3.0
Concentration*	+	±	o	o	o
Dispersion†	o	±	+	+	+
B. *NaCl-KCl:*					
Per cent NaCl			0.8		
Per cent KCl	0.06	0.08	0.10	0.12	0.15
Concentration	o	o	o	±	+

* The symbols for concentration and dispersion have the following meanings: + = marked effect; ± = partial concentration or dispersion; o = no effect.

† Strips blanched in water before placing in NaCl solutions.

HYDROGEN-ION CONCENTRATION

The Buffer solutions found to be suitable for testing the effect of pH on trout melanophore responses were (1) McIlvaine's (1921) mixtures 0.2 M Na_2HPO_4 and 0.1 M citric acid with a range from pH 3.0 to 8.0 and (2) Sorenson's buffer mixtures (Gortner, 1944)—glycine-sodium chloride and sodium hydroxide, giving a range of pH 6.1–13.1. A sufficient quantity of concentrated NaCl solution was added to bring the Na content of the various mixtures to an equivalent of 0.9 per cent NaCl.

The effect of variations in the hydrogen-ion concentration on the melanophores of skin strips is shown in Table 4, which represents the results of a number of experiments. A pH below 5.0 caused contraction of the pigment cells. Then from pH 5.0 to 9.0 they remained in their normal expanded state. At pH 10.0 there was always a partial contraction, which

was replaced by a definite expansion with increased alkalinity. This latter effect was demonstrated by employing skin strips previously blanched in very dilute solutions of adrenalin—1:100,000 and 1:1,000,000.

HORMONAL INFLUENCES

POSTERIOR PITUITARY EXTRACT

Extracts of the powdered posterior portion of porcine and bovine pituitary glands were prepared according to the standard method described in the U.S. Pharmacopoeia (1942),[6] which consisted in extraction with a dilute solution of acetic acid. The concentrations used ranged from 1 to 10 mg. of powder per cubic centimeter of solution. Attempts to adjust the reaction with dilute NaOH resulted in beginning precipitation at a pH of 5.0. The anticipated melanophore-dispersing effect (Odiorne, 1933; Parker, 1934) was not produced when these pituitary extracts, either acid or adjusted to pH 5.0, were injected under the skin of a trout made pale by previous injection of ephedrine. However, when the same extracts were injected under the ventral skin of a pale trout in a white bucket, there occurred within 4–8 minutes a pronounced dark area, 1 × 2 cm., at the site of injection. This reaction disappeared in about 20 minutes. Pituitary extracts had no apparent effect on isolated skin strips which had been previously paled by immersion in dilute KCl or NaCl solutions. Acid reaction and precipitation of the active principle of the pituitary preparation upon reducing the acidity were limiting factors which interfered with further tests.

TABLE 4

EFFECT OF HYDROGEN-ION CONCENTRATION ON MELANOPHORES OF SKIN STRIPS

Response of Melanophores	pH of Buffer Solution			
	2.0–4.0	5.0–9.0	10.0	11.0–13.0
Concentration	+	o	±*	o
Dispersion	o	o	±	+†

* ± = partial concentration or dispersion.

† Strips blanched beforehand in adrenalin solutions 1:100,000 and 1:1,000,000.

THYROID EXTRACT

The pronounced melanophore-concentrating effect of mammalian thyroid extract when injected intramuscularly or subcutaneously in the live fish was described in a former publication (Robertson, 1949). The same effect was observed when isolated skin strips were placed in extracts of desiccated thyroid gland. Since a considerable body of experimental data on this particular subject has been accumulated, it will be presented in a separate communication.

CORTISONE

Subcutaneous injection of a 1:500 solution of cortisone hemi-succinate[7] produced no visible effect on the dermal melanophores in the normal dark trout or in the pale trout on a white background. Likewise, immersion of skin strips in 1:500 and 1:5,000 dilutions of the same preparation of cortisone was without effect on either the concentrated or the dispersed melanophores.

PHARMACOLOGICAL INFLUENCES

A study was made of certain compounds known to exist in the body which

[6] The author wishes to thank Dr. Oliver Camm, of Parke, Davis, and Company, for a potent posterior pituitary extract made up 10 mg/cc, and the late Dr. Fred Koch, director of the biological laboratories of Armour and Company, for powdered preparation of anterior and posterior bovine pituitaries, and Dr. E. M. K. Geiling, department of pharmacology, University of Chicago, for his guidance in preparing the extracts.

[7] This material was provided by Merck and Company through the kindness of the medical director, Dr. J. M. Carlisle.

are believed to play a role in causing changes in the state of melanophore dispersion. Chief among these were adrenalin and acetylcholine, designated by Parker (1940) as neurohumors causing concentration and dispersion, respectively. Other substances were included in the study as examples of agents which counteract the effect of these naturally occurring compounds or which exhibit analogous pharmacological properties.[8] The results of the tests carried out both *in vivo* and on isolated skin strips are shown in Table 5. Demonstration of dispersing effects was performed in fishes blanched with ephedrine or by a white background. The test solutions were injected subcutaneously in 0.4-cc. amounts. Skin strips were blanched by different agents

TABLE 5

EFFECT OF VARIOUS PHARMACOLOGICAL AGENTS ON MELANOPHORES IN THE LIVE FISH AND IN ISOLATED SKIN STRIPS

Agent	Dilution Employed	Test Object
Concentration		
1. Adrenalin	1:000,000 1:10,000,000	Live fish Skin strip
2. Ephedrine	1:10,000 1:1,000,000	Live fish Skin strip
3. Neosynephrine	1:10,000 1:1,000,000	Live fish Skin strip
Slight Degree of Concentration		
1. Histamine	1:2,000 1:2,000	Live fish Skin strip
2. Ergotamine	1:2,000 1:2,000	Live fish Skin strip
3. Tetra ethyl ammonium chloride	1:1,000 1:1,000	Live fish Skin strip (no effect)
No Effect		
1. Cortisone hemisuccinate	1:500 1:500	Live fish Skin strip
Dispersion		
1. Acetylcholine	1:10,000* 1:1,000	Live fish Skin strip
2. Atropine	1:100,000 1:1,000,000	Live fish Skin strip
3. Pilocarpine	1:1,000	Live fish
4. Physostigmine	1:1,000	Live fish
5. Scopolamine	1:2,000 1:10,000	Live fish Skin strip
6. Benedryl	1:10,000 1:10,000	Live fish Skin strip
7. Pyribenzamine	1:1,000 1:1,000	Live fish Skin strip
8. Priscol	1:1,000 1:10,000	Live fish Skin strip
9. Procaine	1:50* 1:00	Live fish Skin strip
10. Banthine	1:10,000† 1:100,000	Live fish Skin strip

* Generalized darkening, as well as local effect.
† Principally generalized effect.

[8] We wish to thank Dr. Julius M. Coon, department of pharmacology, University of Chicago, for providing a number of the pharmacological agents used and Dr. Irwin C. Winter, G. D. Searle and Co., Chicago, for supplying banthine.

—0.2 per cent KCl, 0.2 per cent NaCl, water, and dilute solutions of adrenalin, ephedrine, or thyroid extract.

The reactions appeared in most instances within less than 5 minutes, were sharply defined, and then persisted for from ½ hour to a number of hours, depending on the agent employed and the concentration.

Certain substances produced a generalized reaction in addition to, or without, a local one. The generalized melanophore-dispersing action of acetylcholine was much more pronounced than its local effect, though the latter was definite (Pl. 1, Fig. 5). On the contrary, adrenalin caused only local concentration of the melanophores even in relatively large doses (Pl. 1, Fig. 6).[9] The effects of both acetylcholine and adrenalin began to appear within a minute or two.

and one—priscol—produced dispersion, whereas all three of the parasympatholytic compounds caused marked melanophore dispersion. The difference between atropine and banthine with respect to local and genéral effects is discussed later.

Another interesting finding brought out by this phase of the investigation is the difference in degree of effect between histamine and the antihistamine drugs. Histamine exhibited only a slight concentrating action, while the antihistamines

TABLE 6

EFFECTS ON MELANOPHORES OF PHARMACOLOGICAL AGENTS ARRANGED ACCORDING TO THEIR ACTION ON THE AUTONOMIC NERVOUS SYSTEM

Sympathomimetic:		*Sympatholytic:*	
Adrenalin	Concentration	Ergotamine tartarate	Concentration*
Ephedrine	Concentration	Tetra ethyl ammonium chloride	Concentration*
Neosynephrine	Concentration	Priscol	Dispersion
Parasympathomimetic:		*Parasympatholytic:*	
Acetylcholine	Dispersion	Atropine	Dispersion
Physostigmine	Dispersion	Scopolamine	Dispersion
Pilocarpine	Dispersion	Banthine†	Dispersion

* Effect slight.
† Beta diethylaminoethyl zanthine-9-carboxylate methobromide (Grimson *et al.*, 1950).

Atropine and priscol produced only localized effects, while two other melanophore-dispersing agents—procaine and banthine—caused generalized darkening as well (Table 5).

Arrangement according to their recognized pharmacological action on the autonomic nervous system as shown in Table 6 brings out certain sharply contrasting effects of these agents on the trout's melanophores. The sympathomimetic compounds all caused well-marked concentration, while the parasympathomimetic produced equally definite dispersion. On the other hand, two of the drugs designated as sympatholytic had little or no effect on the melanophores

[9] Two mg. of ephedrine (0.2 cc. of a 1:100 dilution) injected into a 100-gm. trout produced a marked pallor of the entire fish, which lasted 4–6 days.

—benedryl, pyribenzamine, and priscol—produced a most pronounced melanophore dispersion.

REVERSIBILITY OF MELANOPHORE REACTIONS PRODUCED BY VARIOUS INFLUENCES

As stated earlier, the pallor of the live trout produced by asphyxia from whatever cause changed quite rapidly to the original dark phase, with removal of the cause of the asphyxia. Likewise, changes in color due to light intensity of background were rapidly reversible even after the fish had been exposed for a prolonged period to a white background.

Trout killed and placed immediately in water of between 3° and 10° C. remained dark for about 1 hour, then showed a generalized pallor, which lasted 10–20 minutes and was succeeded by a return to

the former dark color, which at 3°–5° C. remained unchanged for at least 8 hours. Isolated skin strips exhibited a similar phenomenon when placed in 0.9 per cent NaCl solution at 10° C.; but the occurrence of the transient melanophore concentration was delayed for 2–4 hours. However, at 3°–5° C. the strips remained continuously in the dark phase for 8 hours or more.

Temperatures up to and including 25° C. did not inhibit reversibility of reaction of skin-strip melanophores exposed to concentrating or dispersing agents. However, at 30° C. the reactivity of the cells was much depressed, and at 35° C. they appeared to be immobilized.

Alternate concentration and dispersion could be produced repeatedly by successive immersion of skin strips in 0.1 per cent KCl and 0.8 per cent NaCl. Strips blanched in water, 0.1 KCl, or dilute adrenalin regained their dark tint rapidly when placed in a balanced NaCl-KCl solution. A drop of adrenalin solution, 1–10 million dilution, placed on the subcutaneous surface of a skin strip will produce a spot of pallor within 1–2 minutes. If the drop is then removed by blotting with filter paper, redispersion of the melanophores occurs within about 15 minutes. Dilute solutions of KCl and also extracts of trout muscle show the same phenomenon. These findings all point to the dispersed state as the normal one for this species of fish.

Certain conditions appear to interfere with the reversibility of the melanophore reaction. Previous exposure to 2 per cent or 3 per cent solutions of NaCl partially inhibited the response of skin-strip melanophores to the contracting influence of adrenalin. A pH of 12.0 had the same effect. Pharmacological agents in too high concentration inhibited return to the opposite state of dispersion, whether spontaneously or under the influence of another agent. On the other hand, with solutions of appropriate strength, contraction and expansion could in most instances be produced once or oftener. Of chief interest in this respect are the observations on adrenalin and acetylcholine, which were carried out most satisfactorily by placing drops of solution on the undersurface of a skin strip. The area of skin under each of two drops of adrenalin (1:10 million dilution in balanced NaCl-KCl solution) exhibited beginning contraction of melanophores within 30 seconds. At the end of 2 minutes the localized areas of pallor were pronounced, and most of the pigment cells were completely concentrated. The fluid was then removed by blotting. On one area a drop of acetylcholine solution (1:100 dilution in 0.2 per cent NaCl) was placed. The other pale area was used for a control. Within 1 minute the melanophores under the acetylcholine showed well-marked dispersion, while those of the control area remained concentrated for 12–15 minutes, and then began to disperse.[10]

Equal parts of the adrenalin and acetylcholine solutions employed above were mixed and a drop of the mixture placed in the skin strip. No concentration of the melanophores occurred, whereas a drop of adrenalin solution placed near by caused the usual pronounced effect. Stronger solutions of adrenalin or weaker solutions of acetylcholine failed to produce these reversing or neutralizing effects.

Complete immobility of melanophores in isolated skin strips was caused by exposure to 1 per cent NaCN.

DISCUSSION

The results of the foregoing experiments, summarized in Table 7, indicate

[10] A drop of 0.2 per cent NaCl placed over the control area made no difference in the rate of spontaneous redispersion.

that melanophores of the dead trout or in skin strips do not respond to certain environmental influences which produce chromatic effects in the live fish, namely, light and asphyxia, both of which act by way of the central nervous system. While the role played by the eye in color changes of teleosts resulting from changes in light intensity of the background has been long recognized and studied extensively, the nature of the asphyxial effect appears to have attracted little attention. One investigator, Lowe (1917), studied the problem by means of bubbling CO_2 through water containing young trout fry. He concluded that the resulting concentration of melanophores exhibited by these trout was due to an increase in CO_2 in their blood. No mention was made, however, of the O_2 content of the water or any changes in reaction. In view of the markedly increased acidity of the water produced in the present experiments by passing CO_2 through it and the finding that this degree of acidity alone would cause melanophore concentration, the changes observed by Lowe may or may not have been due solely to CO_2. While it seems likely that increased CO_2 content of the blood is the cause of the melanophore concentration resulting from asphyxia, adequate evidence is still lacking.

TABLE 7

SUMMARY OF FACTORS PRODUCING CHANGES IN MELANOPHORES OF RAINBOW TROUT*

Factors	Response of melanophores in†	
	Live Fish	Skin Strips
Asphyxia	Conc.	o
Light	Conc.	o
Elevated temp.	Conc.	Conc.
Pressure	Conc.	o
Water	o	Conc.
NaCl <0.5 per cent	–	Conc.
NaCl >0.5 per cent	–	Disp.
KCl	Conc.	Conc.
$CaCl_2$	–	Conc.
pH 5.0–6.0	Conc.	Conc.
pH 5.0–6.0	<6.0	<5.0
pH 10.0–13.0	–	Disp.
Posterior pituitary extract	Disp.	?
Thyroid extract	Conc.	Conc.
Adrenalin	Conc.	Conc.
Acetylcholine	Disp.	Disp.
Histamine	± Conc.	± Conc.

* Including only those pharmacological agents tested which are present naturally in the animal body.

† The meanings of the entries in these columns are as follows: Conc. = concentration; Disp. = dispersion; o = no observable effect; – = not done; ± = partial concentration; ? = could not be determined for technical reasons.

The finding in this study that the asphyxial effect is a central one indicates that the melanophores of the trout possess, for such highly reactive units, considerable independence of changing bodily states. Observations *in vitro* showing that lack of oxygen, high CO_2 concentration, and changes in pH 9.0 to 5.0 apparently do not reduce their activity adds further evidence for this inference.

Melanophore concentration caused by pressure on the surface of the live or dead fish (but not on the isolated skin strip) appears to be due to the liberation of potassium from the adjacent muscle tissue. That potassium can penetrate the subcutaneous tissues readily was shown by the rapid concentration of melanophores occurring in the area under a drop of very dilute KCl or a 0.9 per cent NaCl extract of trout muscle. The redispersion which takes place within 15–20 minutes after removal of the drop of fluid suggests either diffusion of potassium from the pigment cell or possibly neutralization of the potassium effect by a dispersing agent present in the surrounding tissues. That potassium plays a role in the rapid color changes exhibited by the trout in response to variations in background illumination seems improbable, though such an effect cannot be excluded on the basis of our present knowledge.

The observed effects of acetylcholine and adrenaline bear much more directly on this phenomenon. The melanophore-

concentrating effect of adrenalin and the dispersing action of acetylcholine have been shown clearly and repeatedly by a number of workers (Spaeth, 1916; Wyman, 1924, for adrenalin; Parker, 1931; Chin, 1939, for acetylcholine). A further important step in providing evidence for the activity of these substances in the animal body was made by Chin (1939), who found acetylcholine in the caudal fins of the paradise fish, and by Parker (1940), who demonstrated the presence of acetylcholine in the skin of the darkened catfish *Ameiurus* and of adrenalin in the skin of the paled fish of the same species. These substances were isolated in physiologically active amounts. In comparison with the trout, however, the melanophore responses of the catfish to changes in light intensity in particular are very slow. Therefore, to include acetylcholine and adrenalin as possible dispersing and concentrating agents in the trout, the rates of the reactions produced experimentally by them should be of the same order of magnitude as the speed of the color changes resulting from abrupt alterations in background illumination. As a matter of fact, the melanophore concentration produced by adrenalin and the dispersion by acetylcholine both in live fish and in isolated skin strips was fully as rapid as the color changes due to variations in light intensity, all occurring within 1–2 minutes. Furthermore, the finding that application of one of these substances to melanophores previously affected by the other will rapidly reverse the reaction, together with the observation that a mixture of adrenalin and acetylcholine in suitable proportions produces no change in the state of melanin dispersion, is in harmony with the concept of the functional activity of these substances in the body. On the other hand, it should be pointed out that the concentrations employed experimentally, especially with the isolated skin strips, may be quite other than those occurring naturally in the trout.[11]

There is a considerable literature on the response of teleost melanophores to a number of pharmacological substances, which has been summarized by Parker (1948). Many of the results are difficult to interpret because different workers report diametrically opposite reactions with the same drug, even at times in the same species of fish. In the light of the present study it seems likely that some of the contradictory findings may be due to inadequate knowledge of the optimum conditions for functional activity of the particular melanophore species. Space does not permit more than a brief comment on our results. The melanophore-concentrating action of the sympathomimetic and the dispersing effect of the parasympathomimetic agents support Parker's concept of the concentrating and dispersing functions of stimulated sympathetic and parasympathetic nerves, respectively. Certain agents classed in the same pharmacological category were found to differ markedly with respect to the production of local and general effects (see Table 6). Physostigmine and pilocarpine caused only local dispersion, while acetylcholine produced a generalized darkening of the trout. The same difference was exhibited by the parasympatholytic agents, atropine and scopolamine causing a local dispersion and banthine a predominantly generalized one. Are these differences due to a greater diffusibility of certain compounds, or do they reflect activity principally on the autonomic ganglia, as opposed to effects chiefly at the peripheral nerve endings or directly on the pigment cells? These questions suggest a profitable field of in-

[11] Attempts to employ a cholinesterase inhibitor, prostigmine, were not successful, since this substance proved to be quite toxic for trout.

vestigation with this very sensitive test object.

SUMMARY

A study was made of the effect of a number of factors—physiological, physical, mechanical, chemical, hormonal, and pharmacological—which have been found to influence the state of dispersion of the dermal melanophores in rainbow trout. The purpose of the investigation was to ascertain the optimum conditions for maximum reactivity of these cells both in the live trout and in isolated skin strips and to determine the nature of their responses to various environmental and other stimuli.

It was found that the rates of concentration and dispersion of the trout melanophores are among the most rapid recorded for teleost fishes. The normal state appears to be the dispersed phase. The melanophores in the live trout respond to certain influences, viz., changes in background illumination, oxygen lack, and surface pressure, which have no effect on these pigment cells in isolated skin strips. Light and asphyxia exert their action by way of the nervous system. The pressure effect is probably produced by potassium liberated from underlying muscle. Other factors, e.g., temperature, hydrogen-ion concentration, and numerous chemical and pharmacological compounds, produce similar changes in the melanophores of the living fish and in those detached from the body. Reproducible quantitative studies were carried out more satisfactorily with skin strips. The optimum conditions for maintaining the reactivity of such isolated melanophores were a balanced solution consisting of NaCl 0.8 per cent and KCl 0.1 per cent, temperature of 10° C., and pH 5.0–9.0.

With a view to throwing light on the role of the sympathetic and parasympathetic nerves in the trout's response to changes in intensity of background illumination, study was made of the effects of sympathomimetic and parasympathomimetic compounds. The former, including adrenalin, ephedrine, and neosynephrine, produced concentration of the melanophores; the latter, including acetylcholine, physostigmine, and pilocarpine, caused dispersion. Acetylcholine produced redispersion of melanophores concentrated by adrenalin.

Histamine showed only a slight concentrating effect, while the so-called "antihistamine" drugs produced marked dispersion.

Extracts of the mammalian posterior pituitary gland injected subcutaneously were found to produce local dispersion of light-concentrated melanophores. This effect could not be demonstrated *in vitro*. Extracts of mammalian thyroid caused marked concentration of the trout's melanophores both *in vivo* and *in vitro*.

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PLATE I

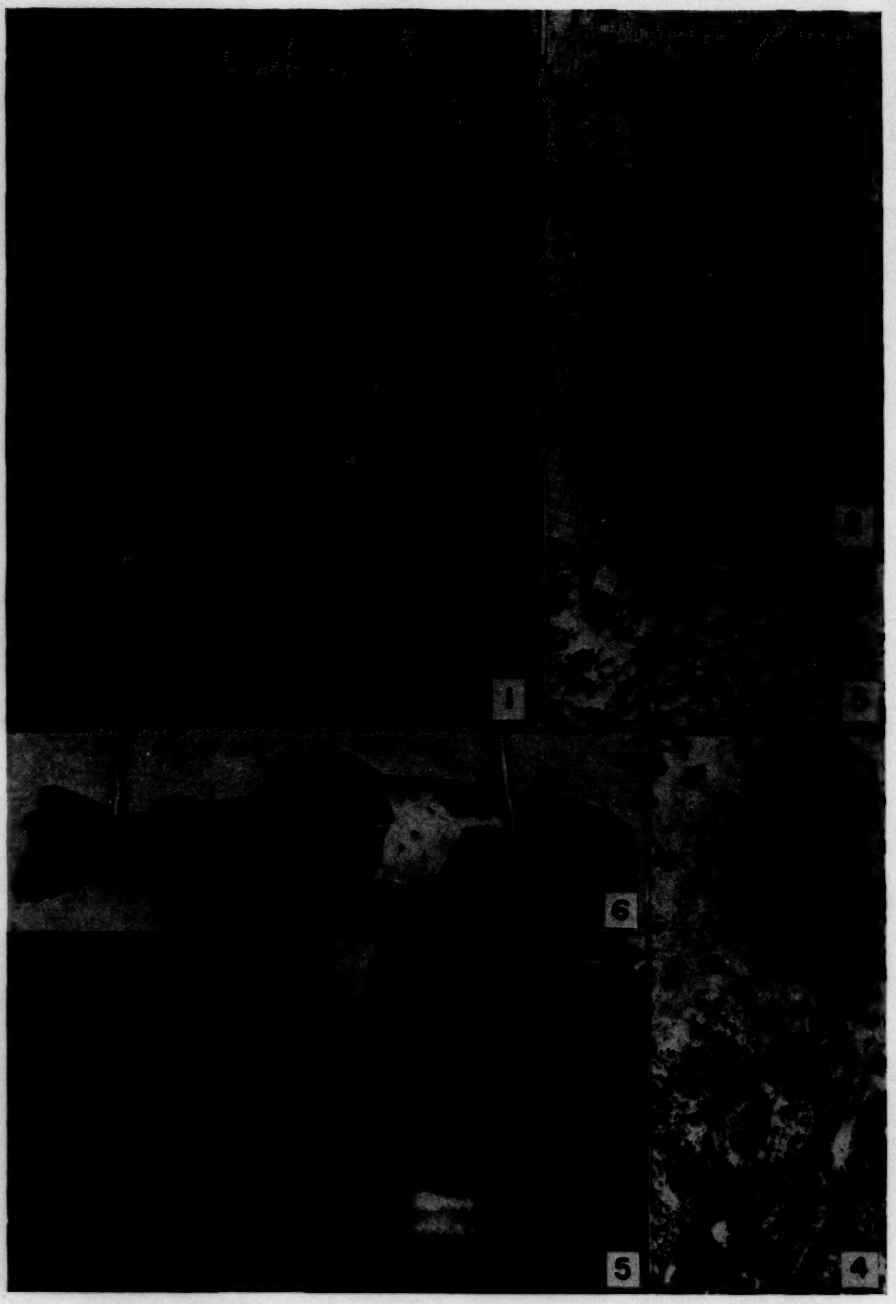

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PLATE I

FIG. 1.—Strip of rainbow trout skin, showing dermal melanophores in normal dispersed state. Magnification ×65.

FIG. 2.—Strip of rainbow trout skin after immersion in N/5 KCl. Most melanophores completely concentrated, but others show only partial concentration, and a few appear to be almost unaffected. These latter are attached to the caudal (external) end of scales. Magnification ×65.

FIG. 3.—Melanophores of subcutaneous tissue in normal dispersed state. Situated just external to muscle layer. Magnification ×65.

FIG. 4.—Subcutaneous melanophores of parr mark which appear as syncytial-like structure seen in upper right-hand part of photograph. However, injection of adrenalin 1:10,000 in this area produced the concentration into discrete cells observed to left of unaffected area. Magnification ×65.

FIG. 5.—Local dispersion of melanophores produced by injection of acetylcholine 1:5,000 under belly skin of trout made pale by exposure to white background. Fish, alive and active, held under water in white enamel pan.

FIG. 6.—Local concentration of melanophores in live trout produced by subcutaneous injection of adrenalin 1:10,000. Light ether anesthesia employed for photography. The greater dispersibility of fluid injected under the skin of the lateral, as opposed to the ventral, surface may account in part for large reaction zone of adrenalin as compared with that of acetylcholine.

THE RATE OF CONDUCTION IN STRETCHED AND UNSTRETCHED NERVES

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IN 1904 Jenkins and Carlson reported that the conduction rate of the pedal nerves of *Ariolimax* remained essentially unchanged when the nerves were stretched. They interpreted these and subsequent data on *Bispira* [*Eudistylia*] (Carlson, 1905) as evidence for a fluid conducting substance in nerve. In view of the large number of more recent investigations in the general field of neurophysiology which tend to localize the nerve impulse mainly in the nerve membrane, these original observations of

[1] Aided by grants from the Medical Research Fund and from the Kinney Fund, Stanford School of Medicine.

Jenkins and Carlson are of even more interest today.

Although there is considerable evidence which correlates fiber diameter with conduction velocity in both vertebrates (Gasser and Grundfest, 1939; Hursh, 1939) and invertebrates (Pumphrey and Young, 1938; Nicol, Smyth, and Whitteridge, 1947), very few experiments concerned directly with the effect of stretch on nerve fibers have been reported. The data from such experiments are scanty and conflicting. Incidental observations by Bullock (1945) indicated that the conduction rate of the giant fibers of *Lumbricus* increased as the fibers were stretched. The experiments of Hagins (1948) tended to support this view. In a brief abstract of a re-examination of the problem, Bullock, Cohen, and Faulstick (1950) report that increase in rate occurs only in a certain percentage (approximately 12 per cent) of the cases examined.[2] Other studies in which the diameter of nerve fibers has been experimentally altered (Hertz, 1947) have shown a *decrease* in conduction velocity accompanying a decrease in fiber diameter.

The data originally reported by Jenkins and Carlson (1904) show great variability. Moreover, in a recent study of the functional organization of *Ariolimax*, Turner and Nevius (1951) found a significant difference between the conduction velocities of the pedal nerves as measured by modern electrophysiological methods and those velocities earlier reported by Jenkins and Carlson (1903). It might be supposed that differences in the degree of stretch of individual pedal nerves could possibly underlie this discrepancy.

In view of the uncertainty of previous data and because of its general pertinence to modern models of nerve and to various theories of conduction, this fundamental problem has been further investigated.

MATERIAL AND METHODS

All the experiments reported here were performed on the pedal nerves of *Ariolimax columbianus*. Specimens of this yellow slug, commonly reaching 8–10 inches in length, were collected in the neighborhood of Stanford University. The extended animals were pinned to a paraffin block and the posterior pedal nerves were exposed (for anatomy see Turner and Nevius, 1951). A measured segment of pedal nerve, 3–4 cm. in length, was secured anteriorly and posteriorly by threads and removed to a small chamber filled with Ringer solution as modified for *Helix* by Cardot (1921). The threads were looped around tie-posts at the ends of the fluid-filled chamber, and by means of these threads the segment of nerve could be stretched to various lengths.

Stimulation of the segment of nerve was carried out through pairs of micromanipulated platinum-iridium electrodes which were coupled through GR 578-A transformers to an electronic stimulator of the general design suggested by Loeffel (1941). In all cases stimuli were condenser discharges of RC 0.5 msec. or longer. The action potentials of the nerve were led by one or more pairs of platinum-iridium electrodes into two differential pre-amplifiers (Grass) filtered to pass without distortion between 0.5 and 10,000 c.p.s. These amplifiers, in turn, fed into a pair of Du Mont oscilloscopes, triggered from the stimulating circuit. The nerve on the recording electrodes was usually raised slightly out of the fluid, while the stimulating elec-

[2] A recent report by Adey (1951) indicates that the conduction velocity of the giant fibers of the Australian earthworm, *Megascolex*, varies in different parts of the worm. Under resting conditions nerve fibers larger than 16μ in diameter show a linear relation between fiber size and conduction velocity in this form.

trodes were kept just beneath the fluid surface. The strength of stimulus was kept constant throughout the course of an experiment, in order to insure that the nerve impulse arose at the same point each time with reference to the stimulating cathode.

The segment of nerve in the bath was usually allowed to assume the shortest length at which no external coiling was apparent. The conduction velocity was then measured by keeping the stimulating electrodes in a fixed position and taking a series of photographs of the action potentials as the recording electrodes were moved to at least three points farther away from the stimulating cathode. The nerve was then stretched to a new length and the procedure repeated. In this way a series of photographs was obtained from which the conduction rates could be calculated by plotting conduction distance against conduction time. In some experiments two pairs of electrodes were set up in parallel, and the distance between them was kept fixed. The time required for a nerve impulse to pass between these fixed electrodes was then measured as the nerve was stretched to various lengths. In all experiments the conduction rate of only the fastest fibers was calculated.

Both stretched and unstretched pedal nerves were fixed in Carnoy's fluid and in 6 per cent formalin in Cardot's solution. The nerves were imbedded by conventional methods, sectioned, and stained with Masson trichrome stain.

RESULTS

More than eighty sets of measurements were made on the pedal nerves of *Ariolimax*. Without exception, the results indicated that these nerves may be stretched to their limits (more than twice their original length) without any significant change in the velocity of conduction of the fastest fibers. The results of a typical experiment are shown in Figure 1. Here the conduction time is plotted against the conduction distance. The conduction velocity is given by the slope of the line, which is seen to remain unchanged, even though the nerve in this case is stretched to five different lengths.

Similar results are obtained when two sets of electrodes are placed in parallel and kept at a fixed distance while the nerve is stretched to different lengths. Thus in Figure 2 it will be seen that the nerve impulse takes the same amount of time to pass between two fixed pairs of electrodes, regardless of the degree of stretch on the nerve. It is of interest to note that in this particular case the general configuration of the action potential changed rather markedly as the nerve was stretched, even though the conduction rate of the fastest fibers remained unaltered. This is a matter of some theoretical importance, since it might be imagined that stretching the nerve could compress the larger fibers, thereby leaving conduction to the smaller (presumably slower) fibers. In other instances, however, the form of the action potential complex can be shown to remain essentially unaltered (Fig. 3).

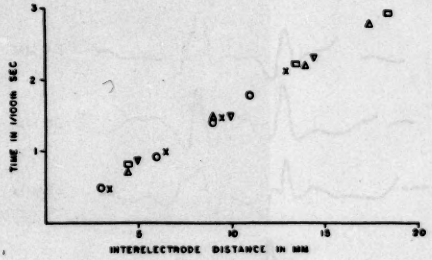

FIG. 1.—Typical graph, showing conduction velocity of a segment of pedal nerve stretched to five different lengths, represented as follows: circles = 18 mm.; *X*'s = 22 mm.; rectangles = 27 mm.; inverted triangles = 33 mm.; triangles = shortened back to 28 mm.

Histologically, the pedal nerves of *Ariolimax* are seen to contain fibers ranging in diameter from 30–35 μ down to 1 μ or less (Fig. 4). The epineurium contains numbers of cells with the characteristics of smooth muscle. As may be

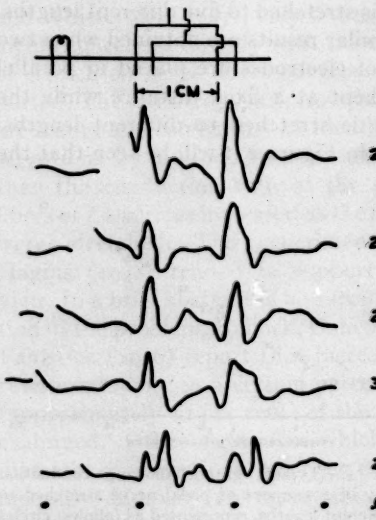

FIG. 2.—With two pairs of electrodes arranged as shown at the top of the diagram, the time taken for the action potential to pass from one pair to the other remains constant, despite different degrees of stretch on the nerve. The different lengths to which the segment of nerve was stretched are indicated to the right of the tracing of the action potential.

seen in Figure 4, *A*, the sheaths of the larger fibers in the relaxed state show considerable wrinkling. Measurements from projections and from camera lucida tracings have shown that the ratio of the distance measured along the irregular membrane to the direct distance measured along the epineurium is of the order of 15.5–12. Since the pedal nerves may be stretched to twice their original length without injury, mere straightening of wrinkled membranes seems inadequate to account for the maximum observed stretched lengths.

DISCUSSION

Several alternative explanations might be considered in relation to the constancy of the conduction velocity in stretched and unstretched nerve. Only the simpler and more obvious need be discussed here.

1. The large internal fibers of the pedal nerve in its unstretched state are seen to possess folded and wrinkled membranes (Fig. 4, *A*). Stretching in this case could, up to certain limits, amount essentially to "unfolding" the fibers, as one would elongate a partially collapsed accordion. The conduction time between any two points marked *on the external surface* (i.e., on the epineurium) of the nerve might then be expected to remain constant

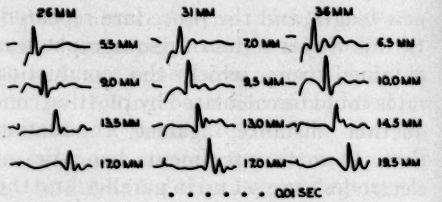

FIG. 3.—The form of the action potential recorded at various distances from the stimulating cathode in a pedal nerve stretched to three different lengths, as indicated by the figure at the top of each of the three columns. The distance from the proximal recording electrode to the stimulating cathode is given by the figure to the right of the tracing of each action potential. Variations in the amplitude of the action potentials are caused by differences in the degree of shunting of the recording electrodes in the Ringer bath.

within wide limits of stretching—the impulse following the irregularities of the membrane and taking a more circuitous course in the unstretched nerve. The *apparent* (i.e., external) distance of the points between which the impulse traveled when the nerve is stretched would appear to be increased. For the conduction rate calculated from external points on the nerve to remain constant, it would be necessary for the nervous impulse in this case actually to slow down as the

nerve is stretched. As has been previously suggested, this slowing could occur if the largest (fastest) fibers in the pedal nerve were progressively compressed and rendered nonfunctional as the nerves were stretched. In view of the fact that the nerve may be stretched considerably without appreciable change in the configuration of the action potential (Fig. 3), this hypothesis is rendered unlikely. Moreover, the discrepancy in size between the largest fibers (only three or four in number) and those of next smaller diameter in the pedal nerves would lead to the expectation of a rather abrupt change in the conduction velocity as soon as the few large fibers were suppressed by compression. No such changes of slope of

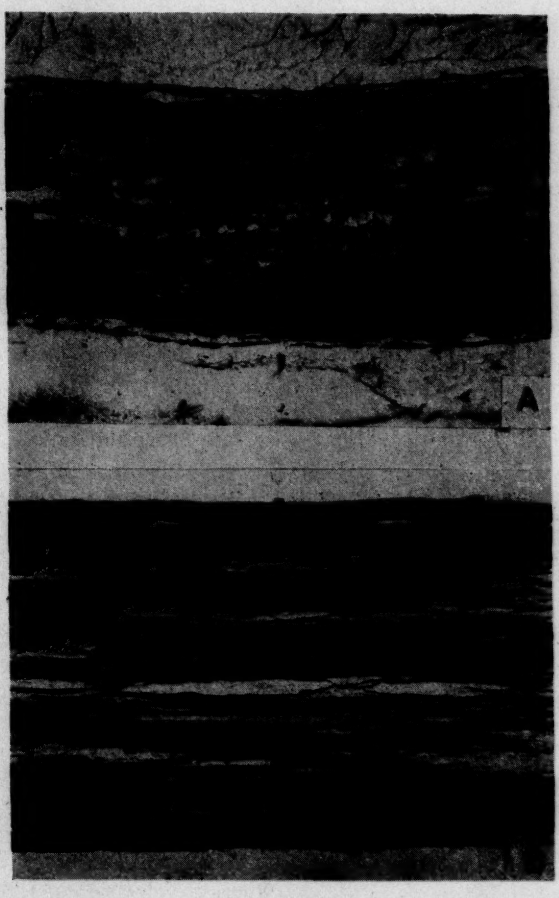

FIG. 4.—*A*, longitudinal section of an unstretched pedal nerve of *Ariolimax*. The membrane of a large central fiber (approximately 35 μ in diameter) shows considerable irregular folding. 100×. *B*, longitudinal section of a moderately stretched pedal nerve. The membrane of the larger fibers is here fairly regular. The large central fiber (approximately 10 μ in diameter), sectioned tangentially, is not the same fiber as shown in *A*. 360×.

any of the curves have been noted. Thus, if real slowing of the conduction rates of individual fibers is postulated to compensate for increased length of nerve due simply to uncoiling or unfolding, some other mechanism than compression of large fibers must be sought.

In those instances in which considerable wrinkling or folding of the nerve membrane is present, the possibility exists that the lines of current flow—transversely through the membrane and longitudinally through the external and internal resistances—may be to a large extent independent of surface irregularities in the membrane. The mere straightening of an irregular membrane might not therefore greatly change the speed of propagation of the action potential along the fiber. The limits of this hypothesis are reached when maximum straightening of the nerve membrane is attained.[3] As has already been indicated, mere straightening of irregularities of the nerve membrane is probably inadequate to account for the maximum amounts of elongation observed in the pedal nerves of *Ariolimax*.

2. The membranes of the internal fibers in the relaxed or partially stretched state may be fairly straight. Stretching in this case might then bring about a real elongation of the fibers, with consequent decrease in their diameter. Because of the variability of fiber diameter along the length of the large fibers of *Ariolimax*, histological techniques (which introduce distortions of their own) have proved unsatisfactory as a means of measuring diameters of stretched and unstretched nerves. In the medial giant fibers of *Lumbricus*, however, Hagins (1948) has demonstrated decrease of fiber diameter —e.g., from 71.0 to 57.0 μ as the fiber was stretched from 2.5 to 5.2 cm. Bullock, Cohen, and Faulstick (1950) also report observations of decrease in fiber diameter with stretch in *Lumbricus*, although no actual figures are given. In such instances the distance between points on the external surface of the nerve (or nerve cord) would be expected to bear a more direct relation to projected points in the interior. It becomes obvious that if the conduction rate measured between external points is to remain the same in the unstretched and the stretched state, the actual conduction velocity would in this instance have to increase, since the actual distance increases.

3. The conduction velocity in stretched and unstretched nerve may remain relatively constant as a resultant of mutually compensating changes in the various characteristics of the nerve fiber—for example, increase in tension of the nerve membrane might compensate for decrease in the radius of the fiber (Fry and Fry, 1950).

4. The possibility cannot yet be wholly dismissed that stretching the nerve, whatever its effect on the shape of nerve membranes, fiber diameters, and other geometrical aspects of the constituent fibers, has no essential effect on the actual velocity of conduction along the fibers. This point of view, although not easily reconciled with many modern models of nerve, is not inconsistent with the data reported here. In such a case, correlations which have previously been made between the spectrum of fiber sizes in mixed nerves and the spectrum of conduction velocities in those same nerves might be regarded as a reflection of underlying structural or qualitative differences (cf. Moore and Turner, 1948). Similarly, in those experiments in which

[3] It may be shown that the *volume* of the nerve fiber is maximum not when the membrane forms a cylinder but when a computable amount of wrinkling is present. I am indebted to Mr. Rollin K. McCombs for a complete mathematical formulation of volume considerations.

changes in conduction velocity and diameters of individual fibers have been brought about by immersing the fibers in hyper- and hypotonic solutions (Hertz, 1947), it is not unreasonable to assume that qualitative changes may have occurred in the fibers as a result of the procedures employed.

SUMMARY

The pedal nerves of *A. columbianus* may be stretched to more than twice their original length without any significant alteration in the velocity of conduction of the fastest fibers. This finding is discussed in relation to the histology of the pedal nerves.

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COMPOSITION OF THE BLOOD OF JAPANESE BEETLE (POPILLIA JAPONICA NEWMAN) LARVAE

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THE present series of experiments was begun at the suggestion of Professor Jean Leclercq, Department of Biochemistry, University of Liége. They are a part of a more extensive investigation designed to discover the biochemical characteristics of insects and of more restricted taxonomical or ecological groups within the class Insecta.

Florkin, Duchâteau, and Leclercq (1949) divided insects into two groups, distinguished by differences in the concentration of sodium in the blood plasma —those in which the sodium index (the percentage of a cation in relation to the

sum of the cations is its index) is high (above 50), and those in which it is low (below 50, and in certain cases in the neighborhood of 1). According to these authors:

> Dans la catégorie à indice de sodium élevé, on rencontre les espèces appartenant à des groups très anciens: Odonates, Mégaloptères, Phryganes, Diptères et Coléoptères. Dans la catégorie à indice de sodium faible, on trouve les espèces appartenant aux groupes qui se sont individualisés au cours du Tertiaire parallèlement au développement des plantes supérieures: l'ordre des Lépidoptères et des Hyménoptères, la famille des Phasmides parmi les Orthoptères, le sous-ordre des Phytophages parmi les Coléoptères. Dans ces groupes, l'indice de potassium est toujours considérable et l'indice de magnésium est encore plus élevé que chez les Insectes de la catégorie à haut indice de sodium.

In the present experiments, a study was made of the concentration of certain inorganic constituents of the blood of Japanese beetle larvae to determine the position of this insect with respect to the above grouping. The experiments were extended to include determinations of (1) some physical properties of the blood and (2) those organic constituents whose concentrations were stated by Florkin (1949) to be characteristic of insect blood.

MATERIAL AND METHODS

Larvae were brought into the laboratory and reared individually in 1-ounce metal salve boxes containing moist soil, with grains of wheat added to serve as food. They were maintained at 25° C. until full grown. Since larvae collected out of doors during the winter months may have a reduced water content, no individual was used until it had been kept in the laboratory at 25° C. for at least 1 week.

Blood was obtained from a larva by removing the tarsus from one of the legs with sharp scissors and allowing the blood to drip into a depression of a porcelain spot-plate while pressure was exerted on the body. The blood, upon exposure to air, gels within a few seconds. Various methods of preventing this gelation were tried. The use of oxalates, citrates, and heparin was ineffective. An attempt was made to anesthetize the larvae with CO_2 (Williams, 1946), but this procedure caused the blood to gel immediately upon exposure to the air. Ether anesthesia, of short duration, was effective in preventing gelation and was employed throughout the entire study. The blood was measured with a 0.1-ml. pipette and from 0.1 to 1.0 ml. was used for each determination, depending on the requirements of the technique. Approximately 30 larvae were required to obtain 1.0 ml. of blood.

The methods used for the preparation of glassware and chemical solutions were the same as those employed by Gese (1950), except that the use of steam from a permanganate bath as a final rinse for glassware was eliminated in most instances. Experience has demonstrated that such treatment is unnecessary.

Water content was determined by evaporation in a vacuum. For each determination, 0.5 ml. of blood was placed in a glass vial previously kept for several days in a desiccator over anhydrous $CaCl_2$. The vial with the blood was weighed and then placed under vacuum desiccation. Weight readings were made daily until constant values were obtained. Water content was calculated from the original readings and those made after complete desiccation.

Osmotic pressures were calculated by Barger's vapor-pressure method. Very small drops of blood were placed in open capillary glass tubes and attached horizontally at their centers with vaseline to the underside of the covers of low Stender dishes. A graded series of NaCl solutions,

ranging from 1.40 to 1.80 per cent, were placed in the Stender dishes, and the covers bearing the capillary tubes of blood were sealed onto the dishes with vaseline. After approximately 15 minutes, the initial size of each drop was measured with an ocular micrometer using a binocular dissecting microscope. Readings were made over a period of 2 days. The size of the drop remained constant only over the isotonic solution. The blood of a single larva was used for each series of determinations. The freezing-point depression of the blood was obtained by placing the isotonic NaCl solution in a Beckmann apparatus and measuring its freezing point. In a similar experiment, blood was placed over graded solutions (0.50–0.58 molal) of urea, a nonelectrolyte, and the concentration of urea isotonic with blood was determined. Osmotic pressures were calculated from these data.

Determinations of the inorganic constituents (calcium, magnesium, sodium, potassium, chlorides, and phosphates) were made, in most cases, by modifications of standard procedures given by Gese (1950). The micromethod for sodium determinations devised by Weinbach (1935) and used by Gese involves analyses on unashed samples of blood. Determinations of sodium in Japanese beetle blood were made on both ashed and unashed blood, and the results obtained by the two procedures were in close agreement. Since Japanese beetle blood has a higher sodium content than the blood of the Cynthia pupa studied by Gese, it was possible to make readings on 0.1 ml. of blood. Japanese beetle blood has a much lower concentration of potassium than the blood of the Cynthia pupa; hence it was possible to use the procedure of Wilde (1939) without the modifications employed by Gese. Because of the low potassium content, 0.5 ml. of blood was needed for each analysis.

The concentration of reducing compounds was determined on samples of 0.1 ml. of blood by the method of Hagedorn and Jensen (see Hawk, Oser, and Summerson, 1947, p. 528). The nonfermentable reducing substances were determined by the following modification of the method of Somogyi (1927). Approximately 1 ml. of dried Fleischmann's yeast and 10 ml. of distilled water were placed in a 15-ml. centrifuge tube and allowed to stand for 2 hours. The yeast was then washed six times with distilled water. After each washing, separation was accomplished by centrifugation. The washed yeast cells were mixed thoroughly in 10 ml. of distilled water, and 2.5 ml. were measured into each of four centrifuge tubes. The yeast in two of these tubes was carried through the procedure as blank tests. To each of the other two tubes, 0.1 ml. of blood was added, and the yeast and blood were allowed to stand for 15 minutes. The reducing compounds remaining after fermentation were determined by the Hagedorn and Jensen procedure. The concentration of these nonfermentable reducing compounds in the blood was obtained by subtracting the amount present in the tubes without blood from that present in the tubes with blood.

Protein nitrogen and nonprotein nitrogen were determined by the micro-Kjeldahl procedure. One-tenth ml. of blood was measured by pipette into a 15-ml. graduated centrifuge tube containing 0.2 ml. of distilled water. The pipette was then washed several times with the water in the centrifuge tube. The protein of the blood was precipitated by the addition of 0.2 ml. of 20 per cent trichloracetic acid. Separation of the precipitate was accomplished by centrifugation, and the precipitate was washed several times with distilled water, the washings being added to the supernatant liquid. Nitrogen de-

terminations were made on each portion. Total nitrogen was obtained by adding the protein and nonprotein fractions.

Amino acid nitrogen was determined by the method of Danielson (1933) as modified by Frame, Russell, and Wilhelmi (1943). This procedure is outlined by Hawk, Oser, and Summerson (1947, pp. 517–19). One-tenth ml. of blood was measured by pipette into a 15-ml. centrifuge tube containing 0.82 ml. of distilled water. The pipette was washed several times with the water in the centrifuge tube. The protein was precipitated by the addition of 0.04 ml. of two-thirds normal sulphuric acid and 0.04 ml. of 10 per cent sodium tungstate. The tube was allowed to stand 5 minutes, and the precipitate was separated by centrifugation. Two-tenths ml. of the supernatant liquid was used for each determination. The photometric determination of amino acid nitrogen was carried out according to the directions in Hawk, Oser, and Summerson. The concentration of nitrogen was obtained by comparing the density of the unknown sample with that of a standard amino acid solution containing 0.03 mg. of nitrogen, using a Coleman Junior spectrophotometer set at 490 mμ. The formula used in this calculation is

$$\frac{\text{Density of unknown}}{\text{Density of standard}} \times 0.03 \times \frac{100}{0.02} = \text{Mg.}$$

of amino acid nitrogen per 100 ml. of blood.

TABLE 1

COMPOSITION OF THE BLOOD OF JAPANESE BEETLE LARVAE

Substance	No. of Tests	Minimum Values	Maximum Values	Average Values
Water	6	90.8 per cent	91.6 per cent	91.2 per cent
Osmotic pressure (as per cent NaCl)	11	1.60	1.70	1.64
Calcium*	5	31.2 mg. per cent	31.9 mg. per cent	31.6 mg. per cent
Magnesium*	5	46.9 mg. per cent	47.4 mg. per cent	47.1 mg. per cent
Chlorides*	5	60.4 mg. per cent	69.3 mg. per cent	67.5 mg. per cent
Chlorides (as NaCl)*	5	99.5 mg. per cent	114.1 mg. per cent	111.2 mg. per cent
Inorganic phosphate*	5	14.9 mg. per cent	15.3 mg. per cent	15.2 mg. per cent
Sodium	9	37.4 mg. per cent	52.3 mg. per cent	46.5 mg. per cent
Potassium	8	31.2 mg. per cent	46.3 mg. per cent	37.2 mg. per cent
Reducing compounds (as glucose)	10	227 mg. per cent	283 mg. per cent	248 mg. per cent
Nonfermentable reducing compounds (as glucose)	8	161 mg. per cent	200 mg. per cent	179 mg. per cent
Total nitrogen	7	1,170 mg. per cent	1,425 mg. per cent	1,302 mg. per cent
Protein nitrogen	7	657 mg. per cent	925 mg. per cent	793 mg. per cent
Nonprotein nitrogen	7	470 mg. per cent	556 mg. per cent	509 mg. per cent
Amino acid nitrogen	8	228 mg. per cent	246 mg. per cent	240 mg. per cent

* Readings made by Robert W. McKinney and submitted by him in a thesis to the Graduate School, New York University, in partial fulfilment of the requirements for the degree of Master of Science.

OBSERVATIONS

The results of the analyses of Japanese beetle blood are shown in Table 1. The water content averages 91.2 per cent. The blood is isotonic to 1.64 per cent NaCl or to 0.54 molal urea solution. Its freezing-point depression, obtained by freezing a 1.64 per cent solution of NaCl, is 1°.03 C. The calculated freezing-point depression, found by multiplying 0.54 by 1°.86 C. (the freezing-point depression of a molal solution of a nonelectrolyte), is 1°.00 C. The osmotic pressure at 0° C., calculated from the freezing-point value of − 1°.03 C., is 12.4 atm.

Japanese beetle blood has a relatively low concentration of inorganic salts and a relatively high concentration of organic

compounds. The concentrations of inorganic ions are given in Table 1 as milligrams per 100 ml. of blood. These values expressed as milliequivalents per liter are: sodium, 20.17; potassium, 9.48; calcium, 15.77; magnesium, 38.76; chlorides, 19.00; and inorganic phosphates, 24.76. The sum of the cations is 84.2 milliequivalents per liter. The sodium index is low (24.0), while the magnesium index is very high (46.0).

The concentration of reducing compounds, expressed as glucose, in the feeding third-instar larva averages 248 mg. per cent. Of this amount, an average of 179 mg. per cent is not fermentable. Hence the average concentration of glucose is 69 mg. per cent. The nature of the nonfermentable reducing substances is not known.

The blood has approximately 1,300 mg. per cent nitrogen. Of this amount, approximately 800 mg. per cent is protein nitrogen. The concentration of protein, calculated by multiplying the concentration of protein nitrogen by 6.25 (the protein conversion factor), is 5.0 gm. per 100 ml. of blood. The concentration of amino acid nitrogen was found to average 240 mg. per cent, or about 50 per cent of the nonprotein nitrogen.

DISCUSSION

The results of these analyses on Japanese beetle blood are in general agreement with the observations of other workers on various species of insects, as given by Wigglesworth (1939), Chauvin (1949), and Prosser (1950). High osmotic pressures appear to be characteristic of the blood of terrestrial insects. The freezing-point depression, 1°.03 C., obtained for Japanese beetle blood, is similar to the values tabulated by Prosser (1950, p. 56) for the blood of four species of insects belonging to three different orders; and the osmotic pressure—12.4 atm. at 0° C.—agrees very well with values listed by Chauvin (1949, p. 220) for various species of Orthoptera. However, Japanese beetle blood appears to differ from that of most other insects thus far studied by its low content of univalent cations. This insect belongs to the group which has a low sodium index. It is a phytophagous coleopteran, and therefore this observation is in agreement with the generalizations of Florkin, Duchâteau, and Leclercq (1949). However, the low potassium index, 11.3, is not in accord with the statement of these workers that those insects with a low sodium index always have a high potassium index.

Bishop, Briggs, and Ronzoni (1925) made a study of the chemical constituents of the blood of the honey-bee larva in relation to their osmotic effects. They found that the most effective fraction of the blood is the amino acids, which have almost twice the osmotic value of the inorganic salts. When the osmotic pressure of other nonprotein nitrogenous compounds was added to that of the amino acids, the total amounted to more than twice the value for the inorganic salts. Comparable calculations for the osmotic effects of the constituents of the blood of the Japanese beetle larva, using data given in Table 1, show that the organic compounds are more than three times as effective, osmotically, as are the inorganic salts. However, this relationship will possibly vary with the nutritive condition of the larva and with its stage of development.

The values reported here for total nitrogen, as well as for the protein, nonprotein, and amino acid fractions, are in excellent agreement with the results of Florkin (1937) for the larva of the silk worm, *Bombyx mori*.

The results obtained in this analysis of Japanese beetle blood are in complete agreement with the generalizations of

Florkin (1949) that insect blood is characterized by high concentrations of amino acids and nonfermentable reducing compounds and by a high magnesium index.

SUMMARY

1. The blood of the Japanese beetle larva has an average water content of 91.2 per cent. It has a freezing point of $-1°.03$ C., and an osmotic pressure at 0° C. of 12.4 atm.

2. The concentration of inorganic cations is relatively low, and that of organic material is relatively high. The organic compounds are more than three times as effective, osmotically, as are the inorganic salts.

3. The sodium index is low, and the magnesium index is high. Contrary to results obtained with other insects which have a low sodium index, the blood of the Japanese beetle larva also has a low potassium index. The average concentrations of inorganic ions, expressed as mg. per cent, are: chlorides, 67.5; sodium, 46.5; potassium, 37.2; calcium, 31.6; magnesium, 47.1; and inorganic phosphates, 15.2.

4. The blood contains an average of 248 mg. per cent of reducing compounds, of which 179 mg. per cent are not fermentable. It contains 1,300 mg. per cent nitrogen, of which 800 mg. per cent are present in the protein fraction, 500 mg. per cent in the nonprotein fraction, and 240 mg. per cent in the amino acid fraction. The average concentration of protein is 5.0 per cent.

5. These observations are in agreement with the generalization that insect blood is characterized by high concentrations of amino acids and of nonfermentable reducing compounds and by a very high magnesium index.

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AMINO ACIDS AND GROWTH FACTORS IN A CHEMICALLY DEFINED MEDIUM FOR DROSOPHILA[1,2]

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As has been realized, a chemically defined medium and growth under aseptic conditions are prerequisites both for exacting studies of the nutrition of organisms and for determining the biochemical difference of genetically different strains. Such studies on multicellular animals have been few in comparison to those on other organisms. That *Drosophila* could be raised in the absence of microörganisms was demonstrated early in the century (Delcourt and Guyénot, 1911; Loeb and Northrop, 1915; and Baumberger, 1919), but a chemically defined medium that will support normal and rapid growth is only now being perfected. However, synthetic media which support growth (slow, but otherwise almost normal, development) of *Drosophila* have been in use in several laboratories (Schultz *et al.*, 1946, etc.; Begg and Robertson, 1948, etc.). Such advance has been made with few other multicellular animals. Much information is available concerning the nutritional requirements of *Aedes aegypti* (Trager, 1935, etc.), but whole casein is still used in the synthetic medium. There is still less information concerning the nutritional requirements of other multicellular animals. Therefore, a more comprehensive study of the medium used for *Drosophila* was suggested.

Some of the nutritional requirements for *Drosophila* were found by Guyénot (1917), Bacot and Harden (1922), van't Hoog (1935, 1936), and Tatum (1939). The first announcement of a chemically defined medium was by Schultz *et al.* (1946) in an abstract, but the quantities of ingredients used were not published. Villee and Bissell (1948) published a formula "essentially similar to Schultz'," using casein hydrolysate. They reported that only about 70 per cent of the larvae pupated and only about 40 per cent became adults, which is far below the normal development. Begg and Robertson (1948, 1950) and Begg (1949) presented a medium (containing casein and gelatin) and stated that it would permit complete, though slow, development. They presented no data to indicate the percentage of pupation or percentage to become adults.

In the light of this paucity of data in the literature regarding a chemically defined medium, it seems pertinent to report at this time the results of a series of studies in this laboratory which have led to a medium, chemically defined (except for agar), which permits growth more rapid than previously reported and which allows all or nearly all the larvae to pupate and become adults.

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MATERIAL AND METHODS

All studies in this series were made with a wild-type strain (Oregon-R) of *Drosophila melanogaster*. Eggs were collected on small flat dishes containing yeast and a corn meal–molasses agar medium from adults in a large glass jar. The eggs were washed in several baths of sterile Ringer's solution and several baths of 70 per cent alcohol, and, when free from all visible particles, they were placed in fresh 70 per cent alcohol. After 45 minutes they were inoculated by means of a flattened wire into sterile tubes of the synthetic medium, using standard bacteriological precautions.

The media used in the various sets of experiments differed (though consistent within a set of experiments), since any improvements discovered in one would be incorporated in the next. The various media are listed in Table 1, and are referred to in the text by their appropriate numbers.

In the preparation of the medium, the amino acids (except cystine) and sucrose were weighed in amounts designed for 100 ml. of final medium, ground together in a mortar, and dissolved in 50 ml. of distilled water, which included the amount of NaOH necessary to neutralize the solution. To this were added, from stock solutions, nucleic acid, the phosphates, the chlorides, the sulphates (except $FeSO_4$, which was made up each time), and the growth factors (except pteroyl glutamic acid, which was in 20 per cent alcohol). The stock solution of the growth factors was made anew each week. Next were added the cystine (dissolved in 4 ml. of 1 N HCl), $FeSO_4$, and pteroyl glutamic acid. The solution was again neutralized and the total volume brought up to 100 ml. with distilled water. Agar was then added and the mixture heated to the boiling point. The medium was then pipetted in 4-ml. amounts into test tubes containing cholesterol. Finally, the tubes were plugged with nonabsorbent cotton, autoclaved for 15 minutes at 15 pounds pressure, and slanted until solidified.

Ten eggs were inoculated per tube, and the tubes were kept at 25° C. (except where noted). Twenty-four hours after inoculation the number of larvae per tube was recorded. Larval counts were made again on the fifth day, and each subsequent day until pupation and eclosion were completed. The tubes were then tested for sterility by adding nutrient broth, incubating them for 48 hours at 37° C., and then streaking some of the contents onto sterile nutrient agar plates, which were then also incubated. Any tubes proving to have a contamination were not considered in the results.

The over-all picture of growth was measured by considering the percentage of the original number of larvae which pupated, the average time in days from egg to pupa, and the percentage of pupae to hatch into adults. Other factors were not considered, since the percentage of eggs to hatch is fairly constant regardless of the medium, as is the time spent in the pupa case. Measurements have been made of larval length and width and of adult weight, but the variation within a single tube was frequently as great as the average variation between experimental sets.

TRYPTOPHANE

Drosophila larvae will not survive without tryptophane (Lafon, 1939). To study this requirement further, dose responses to DL-tryptophane and L-tryptophane (the form of this substance found in nature) were run, with concentrations ranging from 0.003 mg/ml up to 15.0 mg/ml added to a medium which contained only 12 other amino acids (No. 1, Table 1). At the lower concentrations

TABLE 1

COMPOSITION OF VARIOUS SYNTHETIC MEDIA

Material	No. of Medium				
	1	2*	3	4	5
	Mg/Ml				
Alanine			L—1.085	S	
Arginine	L—0.794	S	S	S	S
Aspartic acid			L—1.221	S	
Cystine	L—0.400	L—0.480	S	S	S
Glutamic acid	L—4.418	S	S	S	S
Glycine	0.387	S	S	1.745	0.387
Histidine	L—0.484	S	S	S	S
Hydroxyproline			L—0.384	S	
Isoleucine	DL—1.258	S	L—1.260	S	DL—1.258
Leucine	L—2.345	S	S	S	S
Lysine	L—1.337	S	S	S	S
Methionine	DL—0.339	S	S	L—0.339	DL—0.339
Phenylalanine	L—1.008	S	S	S	S
Proline			L—1.682	S	
Threonine	DL—0.756	S	S	S	S
Tryptophane	L—0.349	S	S	L—1.745	L—0.349
Tyrosine			L—1.240	S	
Valine	DL—1.355	S	DL—1.355	L—1.355	DL—1.355
Sucrose	5.0	S	S	7.5	5.0
Cholesterol	0.1	S	S	S	S
Ergosterol				1.0	
Lecithin	0.1	S			0.1
Ribonucleic acid	1.0	3.0	1.0	S	3.0
Inosine				0.25	
Thymine				0.004	
	μg/Ml				
Biotin	0.0094	S	0.020	S	0.0094
B_{12}	0.004	S	S	0.04	
Ca-pantothenate	6.0	S	S	S	S
Choline chloride	20.0	S	S	S	S
Pteroyl glutamic acid	6.0	S	S	S	S
Pyridoxine	30.0	S	3.0	S	S
Riboflavin	2.4	S	S	S	S
Thiamine	1.5	S	S	S	S
Protogen	2.5 units	S			
B_T	9.375				9.375
Inositol	42.0	S	S		42.0
p-Aminobenzoic acid	2.0	S	S		2.0
Niacinamide	10.0	S	S	S	S
Constants†					

* "S" indicates that the amount is the same as that shown in the preceding column.

† All media contained the following:

Salts (Tatum, 1939):	Mg/Ml
$MnSO_4 \cdot 4H_2O$	0.246
$MgSO_4 \cdot 7H_2O$	0.0129
$FeSO_4$	0.0129
KH_2PO_4	0.606

Salts (Tatum, 1939):	Mg/Ml
K_2HPO_4	0.606
NaCl	0.0129
$CaCl_2$	0.0129
Agar	15.0

there was a distinct level below which there was no growth. With L-tryptophane, this point was in the vicinity of 0.06 mg/ml; with DL-tryptophane, about 0.15 mg/ml. Fair growth (pupation in 14 days) was achieved at concentrations of L-tryptophane slightly less than 0.1 mg/ml and continued to show some improvement up to about 1.5 mg/ml (pupation in about 11.5 days); with DL-tryptophane, fair growth was obtained at concentrations slightly less than 0.2 mg/ml, and very slight improvement up to about 6.0 mg/ml (12.5 days). With L-tryptophane there was no significant change in growth up to concentrations around 3.0 mg/ml, but in concentrations higher than that the dose-response curve began to fall (the length of time necessary for pupation increasing) until, at a concentration of 9.0 mg/ml, 22 days were required for pupation. Higher concentrations would not support growth. With DL-tryptophane there was no significant change in growth up to concentrations as high as 7.0 mg/ml. Concentrations from 9.2 to 15.0 mg/ml were slightly inhibitory (an inhibition was noted by Wilson [1945] with a concentration of tryptophane [DL?] of 4.08–8.16 mg/ml of a medium containing 5 per cent yeast). DL-tryptophane in concentrations above 15.0 mg/ml was not soluble in the medium. Schultz and Rudkin (1948) and Rudkin and Schultz (1949) worked with tryptophane in concentrations of from 0.06 to 0.23 mg/ml. However, they did not state which form of tryptophane was used or what was the effect upon growth except as it pertained to the niacinamide requirement or to the comparison of the requirements of mutant and wild type.

It is suggested by the dose-response data that *Drosophila* does not utilize the unnatural form of tryptophane, since, in general, about twice the amount of the racemic mixture is required to elicit the growth response obtained with the natural form.

A marked phenotypic change occurred at concentrations of L-tryptophane as high as 6 mg/ml. All the flies showed abnormalities of various types—for example, tumors throughout the body, deformed heads, wavy bristles, barlike eyes, abnormal tarsi, darker body color, and darkened eyes. The dark eye color was not brown, as Wilson (1945) found when, in addition to yeast, high concentrations of tryptophane were used in the medium. Rather, the eye appeared to contain the usual amount of red pigment which had darkened, as in the case of sepia and some other eye color mutations in *Drosophila*.

In the earlier phases of the work various ingredients were added to a 2 per cent killed yeast medium to test their possible toxicity. It was found then that tryptophane in high concentrations inhibited growth and that high concentrations of ribonucleic acid would overcome the inhibition (Hinton *et al.*, 1949). It was realized later that with yeast in the medium the results were extremely variable and frequently could not be repeated. For that reason those data are not presented and can be used only as a rough approximation of the true situation. Using the same (No. 1) chemically defined medium, an antagonistic action of nucleic acid to the inhibition exhibited by tryptophane was found to exist but not to be so striking. Data are presented in Table 2 to support the contention that the inhibition caused by tryptophane can, at least in part, be overcome by additional amounts of nucleic acid. The medium for the experiment was divided into four parts: Part 1 served as the control (0.349 mg/ml of L-tryptophane and 1 mg/ml of ribonucleic acid); part 2 contained the higher concentration of tryptophane (6 mg/ml); part 3 was the same as part 2, except that it contained, in addition, an

increased amount of nucleic acid (3 mg/ml); and part 4 was like the control except that it contained the increased amount of nucleic acid. As seen in Table 2, the average time of pupation was increased by 4 days when the amount of tryptophane was increased, but was increased by only 1.6 days when the high concentration of nucleic acid was also present.

TABLE 2

PARTIAL RELEASE OF A TRYPTOPHANE INHIBITION BY NUCLEIC ACID

Part	Original No. Larvae	Per Cent Larvae To Pupate	Average Time to Pupation (Days)	Per Cent Pupae To Become Adults
1	40	100	13.2	97
2	45	85	17.1	58
3	40	97	14.8	60
4	30	100	13.3	87

TABLE 3

DOSE RESPONSE TO DL-ISOLEUCINE AT 27°.5 C.
(12 Other Amino Acids in Medium)

Conc. (Mg/Ml)	Average Day of Pupation
0.00	Larvae died
0.05	Larvae died
0.10	Larvae died
0.20	Larvae died
0.40	21.2
1.25	9.6
4.00	12.6
6.25	13.1
8.00	14.1
16.00	16.0
40.00	20.1

ISOLEUCINE

Isoleucine was demonstrated to be a probable requirement for *Drosophila* by Lafon (1939). That it is a requirement is verified as indicated in Table 3 (medium No. 2, Table 1). The dose-response curve (as measured in this case by the average time required for pupation, in days) indicates a narrow range of tolerance by *Drosophila* to isoleucine. Above optimum concentration it became increasingly toxic.

As with tryptophane, there is an indication that the toxicity of isoleucine can be partially overcome with increased amounts of nucleic acid (medium No. 2). The experiment was divided into four parts (Table 4).

TABLE 4

DESIGN OF EXPERIMENT TO TEST THE RELEASE OF AN ISOLEUCINE INHIBITION BY NUCLEIC ACID

Part	Amount of Nucleic Acid (Mg/Ml)	Amount of DL-Isoleucine (Mg/Ml)
1	3.0	1.26
2	3.0	6.66
3	5.0	6.66
4	5.0	1.26

TABLE 5

PARTIAL RELEASE OF AN ISOLEUCINE INHIBITION BY NUCLEIC ACID AT VARIOUS TEMPERATURES

Temp. (° C.)	Degree of Inhibition by Isoleucine (Days)	Amount of Improvement with Increased Nucleic Acid (Days)
19	3.5	−0.3
20	2.8	+0.3
23	2.8	+0.8
25	3.6	+1.2
27.5	3.1	+0.6

An experiment was completed at each of five temperatures ranging from 19° to 27°.5 C. Shown in Table 5 are the degree of inhibition (measured by the average day of pupation) (part 2 compared to part 1) and the improvement in growth occasioned by the addition of increased nucleic acid (part 3 compared to part 2).

Although the differences are not great, they suggest that the antagonistic action of nucleic acid to the inhibition exhibited by isoleucine is affected by temperature. Nucleic acid is most efficient at 25° C., being less so at a higher temperature and becoming completely ineffective at the lowest temperature tested. Similar results were found to exist in another strain of *Drosophila* tested on a different medium (Hinton *et al.*, 1949).

A study of the ability of *Drosophila* to use just the natural isomer of isoleucine, though not extensive, indicates that growth is somewhat improved at the concentration used (1.26 mg/ml, which represents twice the amount of L-isoleucine expected in the same weight of the racemic mixture). The results of the comparison are given in Table 6 (medium No. 3, Table 1).

TABLE 6

COMPARISON OF GROWTH WITH L- AND DL-ISOLEUCINE

Isoleucine	Original No. Larvae	Per Cent Pupation	Av. Time To Pupation (Days)	Per Cent Pupae To Become Adults
DL....	26	92.3	11.5	83.3
L.....	34	97.1	11.1	87.8

GLYCINE

It was discovered that the correct concentration of glycine was of the utmost importance in obtaining good growth. Originally the amount used was selected as an approximation of that in casein (about 0.1 mg/ml of medium). Subsequently, a concentration of glycine of 0.3876 mg/ml was tried. Examination of Table 7 reveals clearly the marked improvement in growth when the concentration of glycine was approximately four times that found in casein (the medium used was like No. 1 in Table 1) except that no B_T was used). However, Table 7 is an average of a number of sets in which B_{12}, protogen, and the concentration of pyridoxine were experimental variables. Since comparable sets showed the same improvement, they are not presented individually.

A dose response to glycine at concentrations of 0 to 4.845 mg/ml of medium in the presence of 17 other amino acids showed the optimal amount to be about 1.8 mg/ml. Three such experiments were run and gave similar results (medium No. 3 for one; No. 4 for the others). Concentrations less than 0.388 mg/ml gave much slower growth, and concentrations above 2.326 mg/ml became increasingly toxic.

TABLE 7

COMPARISON OF TWO CONCENTRATIONS OF GLYCINE

Conc. of Glycine (Mg/Ml)	Original No. Larvae	Per Cent Pupation	Av. Time To Pupation (Days)	Per Cent Pupae To Become Adults
0.0969.....	181	74.5	17.5	67.4
0.3876.....	557	88.2	12.2	93.1

SERINE

Rudkin and Schultz (1949) reported D-serine as toxic and referred to "sublethal concentrations," thereby implying that it killed the organisms in high concentrations. Our work verifies this conclusion. Using 1.26 mg. of DL-serine (a little less than the amount of serine in casein) per milliliter of various media (No. 1; No. 2 plus B_T; No. 5; and each with different amounts of glycine), the growth of 122 larvae was studied. No pupae were formed. In another experiment, 150 larvae were studied (media Nos. 2 and 5, each with 18 other amino acids). Six days after inoculation of the eggs, only 57 larvae remained alive; on

the tenth day, only 1 was left, and it died without pupating. In the control set (without serine) there was 100 per cent pupation and 93 per cent eclosion. Of the 57 larvae still alive on the sixth day in the set with serine, 16 were in twelve tubes containing 1 mg/ml of ribonucleic acid, while the remaining 41 were in nine tubes containing 3 mg/ml of ribonucleic acid. This may indicate that nucleic acid (as in the case of tryptophane and isoleucine) has a slight detoxifying effect.

The effect of L-serine was studied next. At the time, to our knowledge, only one commercial concern offered the natural form. Using this in concentrations of 1.26 mg/ml, about 100 larvae were studied (medium No. 1). Approximately 50 per cent pupated, in an average of 16.3 days, and only 5 pupae became adults (control: about 80 per cent pupation, 12.3 days, and 70 per cent eclosion). This would indicate that either some D-serine or some other impurity injurious to *Drosophila* was included, since some L-serine, obtained later from a different source,[5] was only slightly toxic. The results, using the pure L-serine, are somewhat puzzling (Table 8). If the differences obtained are to be considered significant, they can be explained only by assuming that L-serine (in the amount used) is slightly inhibitory and that the inhibition can be overcome by increased amounts of nucleic acid in the presence of thirteen other amino acids, but not in the presence of eighteen.

[5] For the pure L-serine we are most grateful to Dr. J. Greenstein, of the National Institute of Public Health, Bethesda, Maryland.

TABLE 8

GROWTH WITH AND WITHOUT L-SERINE
(Medium No. 1 or No. 2 with Variables as Noted in Table)

No. Amino Acids in Medium (Besides Serine)	L-Serine (Mg/Ml)	Amount of Nucleic Acid (Mg/Ml)	No. Larvae	Per Cent Pupation	Av. Time to Pupation (Days)	Per Cent Adults
18	0.00	1.0	52	96.2	11.9	90.4
18	1.26	1.0	54	92.6	11.8	87.0
18	1.26	3.0	105	85.7	12.1	80.0
13	0.00	1.0	52	96.2	12.2	88.5
13	1.26	1.0	52	77.6	14.6	71.4
13	0.00	3.0	92	87.6	12.2	83.2
13	1.26	3.0	57	85.2	10.9	81.4

ARGININE AND THE "ORNITHINE CYCLE"

Arginine was reported as an essential amino acid for *Drosophila* by Lafon (1939); this is clearly verified by the present study (Table 9), as all larvae died without arginine on an otherwise complete medium. The concentration of arginine (0.794 mg/ml) used in these experiments is within the range found by Wilson (1944) to be tolerated by *Drosophila*.

Since the place of arginine in the ornithine cycle has been studied in detail from a chemicogenetic point of view in other organisms (Srb and Horowitz, 1944), it seemed of interest to study, in so far as possible, the relationships of arginine in *Drosophila* nutrition. Only a preliminary study has been completed, and it is presented in Table 9. From these data it is indicated that the arginine requirement can be met only in part by ci-

trulline and not at all by ornithine,[6] at least in the concentrations used. The presence or absence of aspartic acid in the medium appears to make no difference.

OTHER AMINO ACIDS

The results of a number of experiments indicate that growth is better when 18 of the amino acids are used than when only 13 are used. The 13 used were the 10 amino acids essential for the rat (and for *Drosophila* [Rudkin and Schultz, 1947]) plus glycine, cystine, and glutamic acid. The 18 used included these 13, plus alanine, aspartic acid, proline, hydroxyproline, and tyrosine. The natural forms were used in these experiments except that the racemic mixture was used in the case of isoleucine, methionine, valine, and threonine (row 2, Table 10). In more recent experiments all the natural forms were used with the one exception of DL-threonine (row 3, Table 10). Row 4, Table 10, shows the results when the natural form of methionine was used with the racemic mixture of the other 3 amino acids under consideration. Thus this set differs from row 2 only in the form of methionine used. Likewise, row 5, Table 10, differs from row 2 only in that the natural form of valine was used. Otherwise the media were as shown in No. 3, Table 1. The use of other natural forms compared to the use of the racemic mixture has been presented in preceding sections. In all cases there appears to be a very slight improvement in growth when the natural form is substituted for the racemic mixture. The improvement (except

TABLE 9

ATTEMPTS TO REPLACE ARGININE
(Medium No. 2, minus Lecithin)

Arginine (Mg/Ml)	Aspartic Acid (Mg/Ml)	Citrulline (Mg/Ml)	Ornithine (Mg/Ml)	No. Larvae	Per Cent Larvae To Pupate	Av. Time to Pupation (Days)	Per Cent Larvae To Become Adults
0.794	.....	.....	.....	42	83.3	12.2	83.3
.....	.....	.....	.....	45	0.0	.....	0.0
.....	1.221	0.794	.....	41	26.8	29.2	2.4
.....	.....	0.794	.....	53	17.0	27.1	3.8
.....	1.221	.....	.....	57	0.0	.....	0.0
.....	1.221	.....	0.794	57	0.0	.....	0.0

TABLE 10

13 AMINO ACIDS COMPARED TO 18 AMINO ACIDS
(Various Combinations of L- and DL-Forms: See Text)

Row	No. Amino Acids	No. Larvae	Per Cent Larvae To Pupate	Av. Time to Pupation (Days)	Per Cent Larvae To Become Adults
1.....	13	1,073	84.5	13.6	75.7
2.....	18	547	91.6	11.8	84.6
3.....	18	29	100.0	11.1	96.6
4.....	18	36	91.7	11.4	93.9
5.....	18	34	97.1	11.2	93.9

[6] We are grateful to Dr. Milton Heinrich, Amherst College, for supplying us with the citrulline and ornithine.

with serine) is not striking enough to warrant the conclusion that the racemic mixtures are highly toxic, as reported by Schultz *et al.* (1946).

Although marked improvement is obtained by using 18 amino acids, no one amino acid when added to the original 13 gave this improvement, and in all but one case growth was slower than when only the 13 were used, as measured in days to pupation (Table 11).

TABLE 11

NONESSENTIAL AMINO ACIDS
(Medium No. 2 plus B_T)

In Addition to 13 Amino Acids	Average Time to Pupation (Days)
None	11.9
L-alanine	12.5
L-aspartic acid	12.1
L-proline	13.1
L-hydroxyproline	12.9
L-tyrosine	11.7

BIOTIN

Schultz *et al.* (1946) found that biotin was one of the factors necessary for the growth of *Drosophila*, previously missing from the synthetic medium. In the present study a dose response to biotin was carried out by growing the larvae at each of 21 concentrations of biotin (Table 12) ranging from 0.0 to 500 mγ/ml (medium No. 2 plus B_T). In the absence of biotin in the synthetic medium, larval growth was retarded or stopped. Most larvae died with only about 10 per cent pupating (requiring about 20 days), and in no case were adults formed (from about 150 original larvae). Concentrations as low as 0.05 mγ/ml of medium had a definite effect upon the larval survival to the eleventh day, but the percentage of larvae which pupated was not increased, nor did any adults emerge. When the concentration was increased to 0.4 mγ/ml, the percentage of pupation was increased, and some adults appeared. The over-all picture of growth continued to improve as the concentration of biotin was increased, until a concentration of biotin of 16.7 mγ was reached. Thereafter, there was no appreciable improvement. No toxic effect of biotin was obtained, even in the presence of 500 mγ/ml. Trager (1948) suggested a possible toxic effect upon *Aedes* in the presence of 166.7 mγ/ml of biotin; Fraenkel and Blewett (1943) reported a toxic effect for *Tribolium* in the presence of 250 mγ/gm of diet. *Drosophila* also differs from these other insects in that the optimal concentration of biotin for it is lower (around 20 mγ/ml as opposed to nearly 50 for *Aedes* and 100 for *Tribolium*); and the minimal amount of biotin is lower for *Drosophila* (4.2 mγ/ml), an amount that did not allow the emergence of *Aedes* adults.

TABLE 12

DOSE RESPONSE OF *Drosophila* TO BIOTIN

Concentration of Biotin (Mγ/Ml)	No. Larvae	Per Cent Larvae To Pupate	Av. Time To Pupation (Days)	Per Cent Larvae To Become Adults
0	116	10.5	19.3	0.0
0.05	8	12.5	30.0	0.0
0.1	10	0.0	.....	0.0
0.4	9	77.8	20.7	11.1
0.5	8	75.0	22.7	0.0
0.8	8	75.0	19.5	25.0
0.9	7	71.4	19.6	14.0
4.2	25	84.0	17.6	40.0
5.0	8	87.5	17.6	37.5
6.7	32	84.4	14.7	62.5
8.0	7	100	16.0	85.7
10.0	27	74.0	15.5	55.6
13.3	34	82.4	14.2	64.7
16.7	63	93.1	12.3	82.8
33.3	39	89.7	13.3	79.5
66.7	45	93.3	11.5	84.0
136.0	45	95.6	11.6	91.1
200.0	39	97.4	11.8	87.2
275.0	41	90.2	12.2	75.6
350.0	54	90.7	11.4	87.0
425.0	58	94.8	11.7	81.0
500.0	60	93.3	11.9	83.3

Trager (1948), working with the mos-

quito (*A. aegypti*), investigated the relationship of biotin and fat-soluble biotin-active substances in the nutrition of that organism. He found lipid compounds could at least partially replace biotin, suggesting the importance of biotin in their synthesis. It seemed of interest to study such a relationship in another insect and in the absence of casein. Experiments similar to, but not identical with, those designed by Trager were carried out. Lecithin was chosen as the lipid to be used, as it was one of those tested by Trager and has been reported by Delcourt and Guyénot (1911) as having a stimulatory effect upon the development of *Drosophila*. The summarized results in Table 13 (medium No. 2 plus B_T, Table 1) show that, in the absence of biotin, lecithin even in amounts as high as 400,000 mγ/ml allowed no better growth than was obtained in the absence of both lecithin and biotin. A sparing action of lecithin could not be demonstrated, since growth was not improved with concentrations of lecithin as high as 400,000 mγ/ml in the presence of a minimal amount of biotin (4.2 mγ/ml). Nor could any stimulation of growth by lecithin be demonstrated, even with concentrations of lecithin as high as 400,000 mγ/ml in the presence of an optimal amount of biotin (16.7 mγ/ml). Since lecithin showed no activity, it can be concluded that the sample used was biotin-free.

Since the work of Koser *et al.* (1942), of Stokes *et al.* (1947), and of others has demonstrated an interrelation between biotin and aspartic acid and since biotin has been shown to play a role in the conversion of ornithine to citrulline (McLeod *et al.*, 1949), the possibility existed that the failure here to demonstrate the partial replacement of biotin by lecithin was due to the failure of one of these other biochemical cycles. In the light of this possibility, experiments were run to test the ability of lecithin to replace biotin when aspartic acid and citrulline were present in the medium (No. 2 plus B_T). The results (Table 14) fail to show that, in the nutrition of *Drosophila*, biotin is involved in the synthesis of lecithin. There is, however, an indication that citrulline can spare biotin, since, when citrulline was present in addition to a suboptimal amount of biotin, growth was significantly improved. The data are not

TABLE 13

EFFECT OF LECITHIN IN THE ABSENCE OF BIOTIN AND IN THE PRESENCE OF MINIMAL AND OPTIMAL AMOUNTS

Lecithin (Mγ/Ml)	No Biotin				Biotin (4.2 Mγ/Ml)				Biotin (16.7 Mγ/Ml)			
	No. Tubes*	No. Pupae	Av. Time	No. Adults	No. Tubes*	No. Pupae	Av. Time	No. Adults	No. Tubes*	No. Pupae	Av. Time	No. Adults
0	18	36	19.1	0	9	77	17.3	10	3	26	11.9	24
5,000	5	18	21.2	0	6	43	17.2	17	.....	.....	.....	.....
15,000	6	18	20.4	0	6	49	17.3	22	.....	.....	.....	.....
30,000	14	12	24.0	0	7	64	18.2	19	3	28	12.3	22
100,000	4	0	.....	0	3	29	18.6	18	.....	.....	.....	.....
120,000	2	0	.....	0	3	28	19.0	13	.....	.....	.....	.....
150,000	4	3	23.3	0	.....	.....	.....	.....	4	31	12.3	28
200,000	4	1	24.0	0	6	37	18.3	11	2	16	11.9	15
400,000	6	5	19.5	0	6	48	18.3	16	5	52	14.2	41

* Approximately 10 eggs per tube.

complete enough to attempt an explanation of the three adults (5.1 per cent of the original larvae) which eclosed in the absence of biotin when aspartic acid was in the medium (row 2, Table 14). These were the only adults to appear in any experiment where biotin was absent from the medium.

being six other variables involved in the total series of experiments. The results show that, regardless of the medium used, in each of the sixteen comparisons the lower concentration of pyridoxine allowed faster growth and the average time of pupation was less in every case (from 0.3 to 2.0 days faster—one case

TABLE 14

EFFECT OF LECITHIN IN THE ABSENCE OF BIOTIN AND IN THE PRESENCE OF MINIMAL AND OPTIMAL AMOUNTS AND WITH ASPARTIC ACID OR ASPARTIC ACID AND CITRULLINE IN THE MEDIUM

Lecithin (Mγ/Ml)	No Biotin				Biotin (4.2 Mγ/Ml)				Biotin (16.7 Mγ/Ml)			
	No. Larvae	No. Pupae	Av. Time	No. Adults	No. Larvae	No. Pupae	Av. Time	No. Adults	No. Larvae	No. Pupae	Av. Time	No. Adults
	Aspartic Acid (1.2 Mg/Ml)											
200,000	51	3	19.8	0	56	51	18.5	15	.....	.....	.....	.....
400,000	59	14	18.4	3	53	50	18.2	14	.....	.....	.....	.....
	Aspartic Acid (1.2 Mg/Ml) and Citrulline (1.2 Mg/Ml)											
0	29	1	21	0	23	21	15.4	11	41	40	12.2	37
30,000	36	1	24	0	45	40	15.0	27	40	38	13.0	34
100,000	36	6	18.2	0	38	37	14.9	23	45	41	13.1	36
200,000	42	5	19.9	0	39	36	14.6	24	40	37	12.7	28
400,000	40	6	15.0	0	41	38	15.5	25	46	43	13.0	39

PYRIDOXINE

In the light of the findings of Kidder and Dewey (1949*b*), working with *Tetrahymena*, that pyridoxine was necessary in relatively large amounts for optimum growth, experiments were carried out to test higher concentrations with *Drosophila*. In sixteen pairs of sets of tubes a comparison was made of the effect on growth of concentrations of pyridoxine of 3.0 and 30.0 μg/ml of medium. Each set of tubes within a pair of sets contained exactly the same medium except for the difference in the amount of pyridoxine. Any pair might differ, however, from other pairs as to the basal medium used, there

based on only 7 larvae was 3.3 days faster and is not included—with a total average for all sets of 1 day faster, based on a total of 1,080 larvae).

Pyridoxine has been reported as a requirement for *Drosophila* (Tatum, 1941). In the present work dose responses to pyridoxine have given inconsistent results. It is conceivable that some nucleic acids carry contaminations of the growth factor and this possibility is being tested.

VITAMIN B_{12}

When 0.004 mγ of vitamin B_{12} were added per milliliter of medium, there was a consistent, though small, improve-

ment in growth (Table 15). This is true in the ten pairs of sets of tubes available for comparison. An examination of the data reveals that the improvement in growth was probably due solely to a beneficial effect of B_{12} for pupation. (A pair is considered as two sets of tubes, identical except that one contains B_{12} and the other does not. The medium in one pair, however, may contain some factors not present in the medium of the other pairs.) When the average times of pupation (days after inoculation) in the sets without B_{12} are compared to the time in the sets with B_{12}, the two can be seen to be the same. They are always within $\pm$ 1 day of each other. The same is true if the percentage of pupae to become adults is considered. The variation is in both directions. However, when the same consideration was given to the percentage of larvae to pupate, it was found that a higher percentage pupated in each case in the set with B_{12} added (except in 1 case, where the difference was only 1.8 per cent). This higher percentage of pupation with B_{12} added is considered significant, since it was so consistent.

TABLE 15

COMPARISON OF GROWTH WITH AND WITHOUT VITAMIN B_{12} IN THE MEDIUM

With B_{12}				Without B_{12}			
No. Larvae	Per Cent Pupation	Av. Time (Days)	Per Cent Pupae To Beccme Adults	No. Larvae	Per Cent Pupation	Av. Time (Days)	Per Cent Pupae To Become Adults
25	100	16.9	68.0	13	76.9	15.9	60.0
26	92.3	18.1	58.3	17	82.4	19.1	14.3
24	95.8	16.6	52.2	10	80.0	18.5	37.5
19	84.2	16.9	56.3	37	78.6	17.6	40.9
34	100	16.9	47.1	38	94.7	16.2	47.1
35	94.3	18.0	45.5	30	90.0	18.2	55.6
63	88.9	12.4	96.4	51	80.4	13.6	85.4
62	99.7	16.4	93.2	29	86.2	12.0	94.3
26	92.5	18.1	97.9	53	94.3	12.0	94.0
34	100	16.9	71.4	48	83.3	16.1	80.0
Av.	94.8	16.7	68.6	Av.	84.7	15.9	60.9

PROTOGEN

Since protogen,[7] the growth factor so named by Stokstad *et al.* (1949), was found to be essential to *Tetrahymena* (Dewey, 1941, 1944; Kidder and Dewey, 1949*a*), it seemed advisable to see whether it was a factor required by *Drosophila*. The results of ten sets with protogen added (0.0025 units/ml) compared to a comparable set in each case lacking protogen (Table 16) indicated that protogen is not required by *Drosophila*, nor does the addition of it to the medium have any influence on growth, under the conditions of the experiment. It can be noted in Table 16 that only a small variation plus or minus was obtained when the percentage of larvae to pupate, the percentage of pupae to become adults, or the average time to pupation was considered. Since there was no consistency in these results and since the improvement shown by some nearly cancels the decrease shown by others, the foregoing conclusion was drawn.

The possibility remained that the agar

[7] We are grateful to Dr. G. W. Kidder, Amherst College, for supplying us with protogen.

furnished a source of this growth factor. This possibility was tested by comparing the growth of larvae raised on standard Bacto-Agar, as ordinarily used, and on dialyzed Bacto-Agar. Table 17 gives the results, which indicate no significant differences (medium No. 4, Table 1).

TABLE 16

COMPARISON OF GROWTH WITH AND WITHOUT PROTOGEN IN THE MEDIUM

With Protogen				Without Protogen			
No. Larvae	Per Cent Pupation	Av. Time (Days)	Per Cent Pupae To Become Adults	No. Larvae	Per Cent Pupation	Av. Time (Days)	Per Cent Pupae To Become Adults
37	78.6	17.6	40.9	17	82.4	19.1	14.3
38	94.7	16.2	47.1	10	80.0	18.5	37.5
28	92.9	16.4	46.2	25	100	16.9	68.0
19	84.2	16.9	56.3	26	92.5	18.1	58.3
34	100	16.9	47.1	24	95.8	16.6	52.2
35	94.3	18.0	45.5	18	100	17.8	50.0
63	88.9	12.4	96.4	75	87.0	12.9	93.1
35	74.3	17.4	65.4	48	83.3	16.1	80.0
88	91.1	12.2	92.5	53	94.3	12.0	94.0
51	80.4	13.6	85.4	29	86.2	12.0	94.3
Av.	87.9	15.8	62.3	Av.	90.2	16.0	64.2

TABLE 17

TEST FOR PRESENCE OF PROTOGEN IN AGAR

	No. Larvae	Per Cent Pupation	Av. Time (Days)	Per Cent Adults
No protogen, dialyzed agar	37	94.6	10.4	100
Progoten,* dialyzed agar	32	81.3	10.8	100
No protogen, standard agar	28	96.4	10.9	96.3
Protogen,* standard agar	23	87.0	10.6	100

* 0.0025 units/ml.

Fraenkel and Blewett (1947) found a factor necessary for the growth of the insect *Tenebrio* and called it "B_T." It was suggested that B_T might also be stimulatory for *Drosophila*, and it was therefore tried in the medium in eight sets of tubes, each set having a control set containing the same medium but minus B_T.[8] In five pairs (116 larvae), the set containing the B_T concentrate (9.9 γ/ml) was slower to pupate than was the control set (107 larvae) (0.5, 0.6, 1.1, 2.6, and 2.7 days slower, respectively). The other three sets (63 larvae) were faster than their controls (79 larvae), but only by 0.1, 0.3, and 0.3 days, respectively. This is interpreted to mean that B_T at the concentration used has a slight inhibitory effect on the rate of growth of *Drosophila*. However, as with B_{12}, the percentage of larvae to pupate was higher when the B_T concentrate was present in the medium, but the percentage of pupae to eclose as adults was not significantly different. The percentage of pupation with B_T was from 3.1 to 11.9 higher in seven of the

[8] We are grateful to Dr. G. Fraenkel, of the University of Illinois, for furnishing the B_T concentrate and suggesting the concentration to be used.

cases (average increase was 7.8 per cent); a single case was less by 4.2 per cent. The percentage of pupae to become adults was higher in the set containing B_T in three cases and lower in four cases, indicating that B_T had no effect on eclosion. Despite the indication that the B_T concentrate stimulates pupation, its use is not being continued, since it seems to retard larval development and since B_{12} alone seems to stimulate pupation as much or more.

OTHER VITAMIN B REQUIREMENTS

Riboflavin is a definite requirement of *Drosophila* (van't Hoog 1935, 1936; Tatum, 1939). Without it in the medium, all larvae die (Table 18). Pteroyl glutamic acid (Schultz *et al.*, 1946) and Ca-pantothenate (Tatum, 1941) are necessary for rapid growth and pupation (Table 18); but most of the larvae do not die immediately in their absence, since they lived, in the case of Ca-pantothenate, for 29 days. When choline is omitted, growth proceeds at a normal rate, but no pupation occurs. Inositol seems to be unnecessary, no difference in growth resulting because of its presence or absence in the medium (Table 18). Since growth and development are improved when para-aminobenzoic acid is omitted, it can be concluded that this factor is not essential or stimulatory to *Drosophila*. It is assumed that Begg and Robertson realized this, since they do not list the factor in the most recent report of their medium (1950). Niacinamide (Tatum, 1939) and thiamine (van't Hoog, 1935, 1936) have each been reported as requirements for *Drosophila*. Our results agree with their findings (Table 18).

TABLE 18

SOME GROWTH FACTORS
(Medium No. 3 minus B_{12})

Omitted from Medium	No. Larvae	Per Cent Pupation	Av. Time to Pupation (Days)	Per Cent Pupae To Become Adults
None	39	94.9	10.5	100.0
Para-aminobenzoic acid	26	100.0	9.0	100.0
Ca-pantothenate	8	0.0	.....	0.0
Choline chloride	11	0.0	.....	0.0
Pteroyl glutamic acid	8	12.5	16.0	0.0
Inositol	30	96.7	10.3	100.0
Niacinamide	20	0.0	.....	0.0
Riboflavin	12	0.0	.....	0.0
Thiamine	37	0.0	.....	0.0

UNIDENTIFIED FACTORS

Tatum (1939) and Schultz *et al.* (1946) reported a still unidentified factor for rapid development, present in the water-insoluble fraction of yeast. Begg and Robertson (1950) found an alkali-soluble fraction necessary for completely normal development. Since the medium described here admittedly does not support such rapid growth as does yeast (Table 19), several yeast fractions[9] were added to the medium (Nos. 1 and 4, Table 1) to study the effect of a possible unidentified factor lacking in the medium. One yeast fraction (A) was the filter cake from

[9] We are grateful to the Difco Laboratories and to Dr. H. W. Schoenheim, director of the bacteriological laboratories, Difco Laboratories, for providing the yeast fractions and the descriptions of them.

the preparation of Bacto-Yeast Extract left in the press following the first extraction of the autolyzed yeast. Another sample (B) was the residue from the second extraction of the autolyzed yeast. Both residues were dried *in vacuo* and powdered. These were prepared by the Difco Laboratories in the same manner as those they furnished Tatum (1939).

It may be seen (Table 19) that improvement was achieved by the yeast fraction when 12 individual amino acids were used or when Stearns's "parenamine"[10] was used as the amino acid source. However, in the presence of 18 individual amino acids, development was somewhat accelerated with fraction A, which contains, in addition to the autolyzed yeast cells, a small amount of soluble compounds, but was not accelerated with fraction B, which contains a very small amount, if any, of the soluble compound. This was true on both media Nos. 1 and 4. It may indicate that whatever was supplied previously by such fractions is already present in our medium, which contains 18 amino acids (but lacking or in too low a concentration in "parenamine" and the 12 amino acid mixture), but is not entirely effective because of the imbalance of factors. This would suggest that, when the proper balance of the amino acids is achieved, the present medium may support rapid growth without the addition of unidentified factors.

TABLE 19

UNIDENTIFIED GROWTH FACTORS

No. Amino Acids in Medium	Yeast Fraction	Av. Time to Pupation (Days)
No. 1 12 (no glycine)	None	19.9
No. 1 12	B	13.5
No. 1 12	A	10.2
No. 1 18	None	14.9
No. 1 18	B	14.2
No. 1 18	A	10.5
Parenamine	None	13.4
Parenamine	B	8.8
No. 4 18	None	9.2
No. 4 18	B	9.7
None	Whole yeast	5.4

THE MEDIUM

In the light of the results of all the foregoing experiments and the literature on the subject (Schultz *et al.*, 1946; Begg and Robertson, 1950; Hinton *et al.*, 1950), a medium has been derived which includes those factors shown to be necessary or stimulatory and in amounts as near optimal as possible. This medium is presented as No. 4 (Table 1), and the picture of growth resulting on this medium is presented in Table 20.

TABLE 20

GROWTH ON THE CHEMICALLY DEFINED MEDIUM NO. 4 GIVEN IN TABLE 1

Temp. (° C.)	No. Larvae	Per Cent Larvae To Pupate	First Day of Pupation	Last Day of Pupation	Av. Time to Pupation (Days)	Per Cent Pupae To Become Adults
25	43	97.7	7.0	9.5	7.5	97.6
28	42	85.7	5.5	8.5	6.7	97.2

Actually, this medium is the sum total of all individual improvements found in the various experiments. No attempt has been made to alter each ingredient individually in the present mixture. It is

[10] A modified casein hydrolysate suggested by Dr. Jack Schultz (personal communication).

planned to perform such experiments, and until that time no claim can be made that all ingredients are necessary or in the proper concentration in the medium as it is now constituted. It is only too clearly indicated by the various experiments that the optimal concentration of one ingredient may vary, depending upon the balance of other factors.

DISCUSSION

The development of a chemically defined medium on which *Drosophila* can be raised in the absence of microörganisms has progressed to the point that exacting studies can now be made of the role of various amino acids and growth factors in the nutrition of this insect. This medium supports normal development, and most of the larvae become adults. However, larval development is prolonged for 1 or 2 days, indicating that either some growth-stimulating factor is absent from the medium or that the balance of nutrilites already in the medium is not adjusted for optimal development. The latter hypothesis is being tentatively adopted. It may be recalled (Table 19) that, with 18 amino acids present in the medium as the sole source of amino acids, no improvement in the rate of growth could be demonstrated by the addition of a a yeast fraction found by Tatum (1939) and Schultz *et al.* (1946) to contain an unidentified growth-stimulating factor. The unidentified growth factor continually fails to stimulate growth as the medium becomes progressively refined. Further work is in progress to attempt to adjust the balance, especially of the amino acids. This becomes a still more logical approach, since Begg and Robertson (1950) found an alkali-soluble yeast fraction which, when added to casein, gelatin, and tryptophane as the amino acid source, gave the normal rate of development and which shows nucleoprotein-like reaction.

During the course of the work on the amino acid balance, a relationship of nucleic acid to amino acids was noted. In the case of tryptophane, isoleucine, and serine (the only amino acids tested in this connection), high concentrations of the amino acid resulted in an inhibition of growth which was partially overcome by the addition of higher-than-usual concentrations of nucleic acid. With just nucleic acid in the higher concentration, the growth was usually somewhat inhibited. This relationship existed when only 12 other amino acids were present in the medium. At least in the case of serine, when 18 other amino acids were present, there was a negative effect with the addition of extra nucleic acid. Why such a relationship should exist is difficult to explain. It is conceivable, however, that, in the building of nucleoproteins by the organism, the various amino acids are in some way dependent upon the presence of nucleic acid.

That higher levels of glycine were found to be so important in bettering the growth is not surprising, since Kidder and Dewey (1945) found in *Tetrahymena* that glycine seems to function as a detoxifying agent for the essential amino acids. The high amounts of glycine used in the medium for *Drosophila* would also account for the lack of need for serine which was shown by these experiments (Kidder and Dewey, 1947).

Although more extensive studies are needed and are in progress to study the relation of arginine to other nutrilites in the nutrition of *Drosophila*, the preliminary results reported here indicate an inability to convert ornithine to citrulline. However, these results do show a relationship of biotin, citrulline, and arginine, since arginine may be partially replaced by citrulline and since biotin, in turn, is spared by citrulline.

These studies of the nutritional requirements of *Drosophila* do not reveal

any major deviations from the general nutritional pattern shared by all animals so far investigated. Minor differences are known to exist between the various animals studied (see Kidder and Dewey, 1947, for a comparison of *Tetrahymena* to mammals; and Albanese, 1947, for a review of the mammals). *Drosophila* is like some animals in certain respects and like others in some details. Thus *Drosophila*, like man, apparently cannot utilize the D-isomer of tryptophane, while *Tetrahymena* and the rat can. *Drosophila* differs from some animals, since it apparently does not complete the ornithine cycle and in this respect is more nearly like *Tetrahymena*. Insects differ from *Tetrahymena* in that they require biotin, but *Drosophila* differs from the other insects studied under comparable conditions in its quantitative requirements and its tolerance for biotin and the possible biotin-lecithin relationship. *Drosophila* differs from some microörganisms which are able to dispense with biotin in the presence of aspartic acid (Koser *et al.*, 1942; Stokes *et al.*, 1947) but is like some other organisms in that biotin seems to play a role in citrulline formation (McLeod *et al.*, 1949). Still other differences and similarities have been pointed out.

Of most general importance for optimal growth of *Drosophila* appears to be the balance of nutrilites, and this, undoubtedly, is a highly complex set of interrelationships, as was shown for *Tetrahymena* by Kidder and Dewey (1947) and suggested by Schultz and Rudkin (1949) and Rudkin and Schultz (1949) and by this study of some of the amino acids and growth factors in the nutrition of *Drosophila*.

SUMMARY

1. A series of experiments was made in an attempt to perfect a completely chemically defined medium for *Drosophila* that will support normal development.

2. The results indicated that *Drosophila* does not utilize D-tryptophane, metabolizing only the natural form. A syndrome of phenotypic abnormalities occurs when the flies are raised on high concentrations of L-tryptophane.

3. Tryptophane, isoleucine, and serine have an inhibitory effect on growth when present in high concentrations, and the data suggest that high levels of ribonucleic acid act as an antagonist to the inhibition, at least when only 13 amino acids are used in the medium.

4. Dose responses to tryptophane, isoleucine, glycine, biotin, and pyridoxine were carried out.

5. D-serine is extremely toxic to *Drosophila;* L-serine is slightly toxic under most circumstances.

6. The arginine requirement can be met in part by citrulline, but not by ornithine in the concentrations used. Biotin is spared by citrulline in the absence of arginine.

7. Glycine in high concentrations has a detoxifying effect on essential amino acids and, as a result, is most important for optimal growth.

8. Better results were obtained with 18 amino acids in the medium than when any other combination was used. The natural forms are generally best, although the racemic mixture gave growth almost as good in the case of some of the amino acids.

9. *Drosophila* tolerates concentrations of biotin over a large range, with no toxic effect demonstrable, as has been reported for some other insects. Nor could lecithin be shown to be able partially to replace biotin.

10. High concentrations of pyridoxine inhibited development. Vitamin B_{12} consistently raised slightly the percentage of larvae to pupate. Protogen could not be

shown to influence development or to be a requirement. B_T improved pupation very slightly but inhibited the rate of development to a small degree.

11. Other B-group vitamins were tested; inositol and para-aminobenzoic acid were found to be unnecessary and, in the case of para-aminobenzoic acid, to be somewhat inhibitory.

12. Various yeast fractions containing possible unidentified growth factors added to a complete medium failed to improve development.

13. A medium is presented which is completely chemically defined except for the agar base and which allows normal development of nearly 100 per cent of the eggs to the adult condition, with pupation occurring in 6–7 days (only 1–2 days slower than the normal). It is suggested that the normal rate of development can be achieved with further studies on the balance of nutrilites in the medium.

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OXIDATION-REDUCTION INDICATOR PATTERNS IN THE DEVELOPMENT OF CLAVELLINA HUNTSMANI

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THE high degree of cytoplasmic organization in ascidian eggs has been the subject of many studies with various techniques and with remarkable agreement, in most respects, as regards the general character and regional localization of the most conspicuous features of the pattern. A general survey of the literature concerned with this organization is beyond the present purpose, but attention is called to certain points in the work of others and their relation to the data presented here. The present paper is concerned with certain features of developmental pattern which are made directly visible by means of oxidation-reduction indicators. Other investigators have used various indicators on other ascidian eggs and on eggs of other organisms (Gersch and Ries, 1937; Ries, 1937, 1939; Reverberi and Pitotti, 1939). As regards the more conspicuous features of pattern, the data of the present paper agree closely with those of the earlier studies, but their techniques failed to show certain other characteristics of pattern which appear clearly and uniformly when the indicators are used as noted in the following section.

MATERIAL AND METHODS

Clavellina huntsmani is abundant at Pacific Grove, and during the summer eggs and developmental stages can be easily obtained. Natural fertilization apparently occurs either at night or very early in the morning, and undivided eggs and early cleavage stages can be obtained during morning hours. It has not been possible thus far to determine more exactly when natural fertilization occurs. Although gonads usually contain full-

grown eggs apparently ready for fertilization, fertilizations during the day have not been successful, in spite of numerous attempts, at both self-fertilization and cross-fertilization, made during each of four summers. Fertilized eggs develop in the laboratory, even in standing water, to advanced larval stages still within the membrane. Changes of pattern during metamorphosis have not been followed.

Modifications of the indophenol or Nadi reaction and the dyes methylene blue and Janus green (diazine green) have been used. It was regarded as desirable to avoid rapid killing of the material by the indophenol reagents or by intracellular indophenol or oxidized dyes, since work with other organisms had made it evident that, with rapid killing, certain features of pattern might be obliterated. It appears from the earlier papers that rapid killing did not alter appreciably the more conspicuous features of developmental pattern, though it apparently obliterated certain other slight regional differentials.

Since the techniques used on the *Clavellina* material and particularly that of the indophenol reaction were very different from that used in the earlier studies, it seems desirable to discuss it once more in some detail. Considering, first, the indophenol reaction as used for *Clavellina*, this reaction consists in the intracellular formation of deep-blue indophenol from the reagents dimethylparaphenylene diamine (para-aminodimethyl aniline) and α-naphthol catalyzed by an oxidase, "indophenol oxidase," regarded by some as cytochrome oxidase or a related enzyme. Regional differences in rate and intensity of the catalyzed reaction are regarded as indicating regional differences in oxidase activity. For this reaction Ries (1937) and Reverberi and Pitotti (1939) used each of the reagents in 1 per cent concentration in distilled water, heat and a small amount of NaOH being used to dissolve the α-naphthol and sea water being removed as completely as possible from the material. Dimethylparaphenylehe diamine is highly toxic in concentrations far below 1 per cent; α-naphthol is also certainly highly toxic in 1 per cent solution with NaOH added and in distilled-water solution. Used in this manner, the indophenol reagents must kill almost at once.

In the use of the indophenol reaction on Protozoa (e.g., Child, 1949), coelenterates, planarians, and annelids and also on teleosts and amphibians, much lower concentrations have been found far more satisfactory than these. The low concentrations permit the reaction to occur in living motile organisms as well as in living embryonic material, and, as has been made evident in various papers, the method, used in this way, constitutes an extremely delicate means of making directly visible even very slight regional differences in certain features of physiological pattern in many organisms. The procedure found most satisfactory in the use of very low concentrations of reagents for the indophenol reaction is as follows: If the diamine is in solid form, slight heating liquefies it. One small drop of the liquefied reagent (approximately 40 drops equals 1 ml.) is added to 10 ml. of salt or fresh water, according to the material, for a stock solution. If the reagent is pure, this solution is approximately 1/400. A solution of α-naphthol is made by adding 1 mg. or less—usually estimated, not weighed—to 10 ml. of salt or fresh water. In a few minutes enough α-naphthol has dissolved to give a good reaction without warming or addition of NaOH.[1] This is approximately

[1] Occasion is taken here to call attention to an error in an earlier paper in the account of preparation of reagents for the indophenol reaction (Child, 1949). In the ninth line from the top of p. 316, "1 ml. or less of α-naphthol" *should read* 1 mg. or less.

1/1,000 or less. For use with living material 1 small drop of diamine and 1–2 drops of α-naphthol solution, added to 1 ml. of water, have usually served as a starting point for determining the concentrations most satisfactory for given material. These concentrations have sometimes been regarded as standard merely as a matter of convenience. The reaction occurs with a wide range of concentrations above and below these, but concentrations more than five to six times above or below these have rarely been used. Susceptibility to toxic effect differs greatly in different organisms, but with most forms thus far used concentrations can be found by trial in which the reaction occurs with ciliary or muscular activity continuing in motile forms and development continuing in nonmotile embryonic stages until intracellular concentration of indophenol becomes high. For *Clavellina*, concentrations of 1, 2, 3, and 4 drops of each reagent in approximately 1 ml. of sea water have commonly been used, and still lower concentrations have also been tested. Variations in concentrations of each reagent were also used in determining the most useful concentrations. With the higher concentrations the reaction was rapid, but evidences of toxic effect appeared rather early. With less than 1 drop of each reagent in 1 ml. of water, reaction was slow but with essentially the same regional differentials. The indophenol reaction with these low concentrations of reagents indicated essentially the same regional pattern of the most strongly marked differentials as the reaction with 1 per cent concentrations of the reagents, as well as the benzidine reaction for peroxidase as used in the earlier work, but it has also brought to light certain very slight differentials not previously recorded. The benzidine reaction has not been used on *Clavellina* because it undoubtedly kills almost immediately, or, with the pre-fixation used in some cases. The material is killed before exposure to the benzidine reagents.

Various vital and other dyes were also used on ascidian developmental material in the earlier studies. Ries (1939) used the so-called "leucobases," i.e., dyes reduced by rongalite. This is a highly toxic reducing agent and must be used with great care and in low concentration to avoid differential toxic effect and error. Staining in solutions of the reduced colorless dyes results from their intracellular oxidation. Use of sodium hydrosulphite for dye reduction is far more satisfactory than reduction by any of the highly toxic reducing agents, such as rongalite or hyposulphite and HCl. Minute quantities of hydrosulphite are sufficient to reduce completely a wide range of dye concentrations of methylene blue, Janus green, and various other oxidation-reduction dyes in 1–2 ml. of water. Reduction occurs in a few seconds, and even in open preparations the deeper levels of the solution may remain reduced for 10–15 minutes or indefinitely, if sealed with exclusion of air. Moreover, this reducing agent is nontoxic even in concentrations far above those required for reduction, although aerobic forms remaining in the reduced solutions for long periods finally die from lack of oxygen. The reduced dye enters most cells very rapidly, and intracellular oxidation is rapid, unless too much hydrosulphite is used, and is without toxic effect until intracellular concentration of oxidized dye becomes high. This oxidation in reduced dye solutions has been called "primary oxidation," in order to distinguish it from reoxidation following intracellular reduction. After intracellular oxidation of reduced dye, differential reduction by hydrosulphite is also possible; and in most material reoxidation and reduction

can be repeated two to three times while the material is still living and motile forms are active. But sooner or later, as intracellular concentrations of oxidized dye increase, reduction is differentially retarded, and regional differentials in the material may be partially or entirely reversed, parts which origina'ly reduced most rapidly reduce last or not at all. Differential reduction of intracellular indophenol by sodium hydrosulphite and its differential reoxidation by oxygen increase is also possible in living active organisms, even in single protozoan cells, with the same differential retardation or absence of reduction as with dyes when the intracellular concentration of indophenol becomes high.

These points concerning the use of oxidation-reduction indicators and of sodium hydrosulphite as reducing agent have been repeatedly stated in the writer's earlier papers but are again called to attention here because, with hydrosulphite as reducing agent, more varied use of the indicators becomes possible than with the highly toxic reducing agents. For example, intracellular oxidation and reduction are often possible more than once in the same material while still living and not visibly injured. It also makes it possible to show the differences in reoxidation pattern with slight or slow oxygen increase, as compared with rapid and great increase following reduction. Differential retardation or absence of reduction after oxidation makes it possible to determine approximately when the intracellular concentration of the indicator becomes toxic. And, finally, the regional differential in toxicity indicates regional differentials in susceptibility to intracellular concentrations of the indicator.

A considerable number of dyes have been used on ascidian material, but the matter of concentrations of dyes used does not seem to have interested the earlier authors. The only oxidation-reduction dyes used on the *Clavellina* material were methylene blue and Janus green (diazine green). Methylene blue 1/10,000 was found by preliminary experiment to give good results. This was used oxidized for direct staining, followed by reduction, in some cases by oxygen uptake of the material sealed in a small cell in a small volume of the dye solution, in other cases by hydrosulphite. Both dyes were also used reduced to "leucobase" by hydrosulphite and used for intracellular oxidation with following reduction: Janus green was used in concentrations from 1/50,000 to 1/10,000 in essentially the same way, though only partial reoxidation to the red form, diethyl safranine, occurred, as in most cases with living material. The oxidation-reduction pattern indicated by the two dyes in the living ascidian material was not essentially different from those shown by the indophenol reaction; but intracellular oxidation of dyes reduced by hydrosulphite and intracellular reduction of oxidized dyes—also by hydrosulphite—provide additional data of interest concerning the patterns of *Clavellina*.

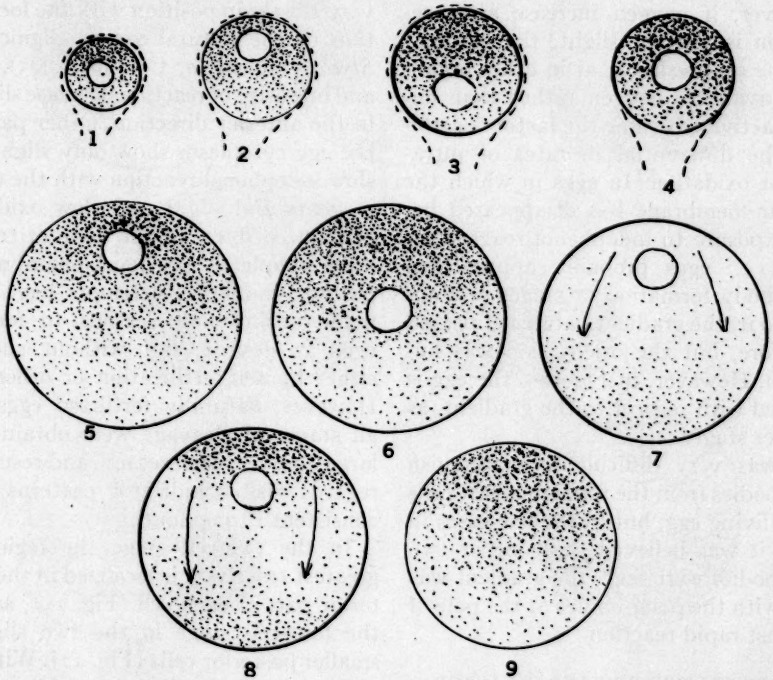
Patterns of oxidative reaction or reduction are indicated in the figures by shading or by arrows pointing in the direction of decrease of reactivity. Longer arrows signify more, shorter arrows less, rapid progress of reaction or reduction.

OÖCYTE STAGES

From very early stages the cytoplasm of the growing ovarian oöcyte is uniform in appearance; but the indophenol reaction, with the low concentrations of reagents used—two to four or even five times the concentration noted above as "standard"—makes visible a distinct cytoplasmic gradient decreasing from or near the region, which may be regarded as the free or freer pole, i.e., the pole op-

posite the short stalk of the ovarian membrane surrounding the cell (Figs. 1 and 2). In these earlier oöcytes the nuclear position in relation to this gradient is variable. In very early stages the nucleus may be approximately central. As growth progresses, it is usually nearer the more rapid and deeper indophenol reaction. Within this half it is usually near the deeply reacting pole (Fig. 5), but occasionally near the center (Fig. 6).

In earlier stages of the indophenol reaction, reduction of intracellular indophenol by hydrosulphite was most rapid



FIGS. 1–9.—Gradient pattern in stages of oöcyte development and full-grown unfertilized eggs. Figs. 1–4, early oöcyte stages with different positions of the nucleus in relation to the gradient. Figs. 5 and 6, nuclear positions in relation to the gradient in full-grown eggs. Fig. 7, differential reduction of indicators preceding alteration of pattern by toxic effect. Fig. 8, differential reduction with retardation or absence of reduction in regions originally of greater reactivity. Fig. 9, gradient pattern in unfertilized egg after disappearance of nuclear membrane.

free pole and in the more rapidly reacting region of the indophenol gradient, as in Figure 2. Oöcytes are occasionally found in which the nucleus is nearer the stalk (Fig. 3) or still approximately central (Fig. 4). In later stages and full-grown egg cells, its position is still somewhat variable, but in all the numerous cases observed it is in the half showing the in the region of most rapid reaction, i.e., the reduction gradient was the same as the reaction gradient. After long-continued reaction—an hour or more—or with the higher concentrations of the reagents, the reduction gradient was more or less completely reversed; the regions originally of most rapid reaction failed completely to reduce (Fig. 8). These gradient

reversals and differential failures to reduce are regarded, as in other organisms, as resulting from differential toxic effect and also indicate graded differential susceptibility to the toxic action. Preceding toxic effect, reoxidation following reduction showed the same gradient as did the primary oxidation in many cases. However, if oxygen increase after reduction is slow or slight, the gradient may be reversed, i.e., as in other organisms, available oxygen rather than enzyme activity became the factor controlling the differential in rates of intracellular oxidation. In eggs in which the nuclear membrane has disappeared before exposure to indophenol reagents or dyes, i.e., eggs probably approaching polar body formation or stages preliminary to it, the gradient pattern appeared as before, but the orientation was uncertain. However, in Figure 9 the egg is oriented with respect to the gradient, as in other stages.

It was very difficult to distinguish polar bodies from the numerous test cells in the living egg, but in the few cases in which it was believed that one or two polar bodies were seen, the gradient was polar with the polar bodies at the pole of the most rapid reaction.

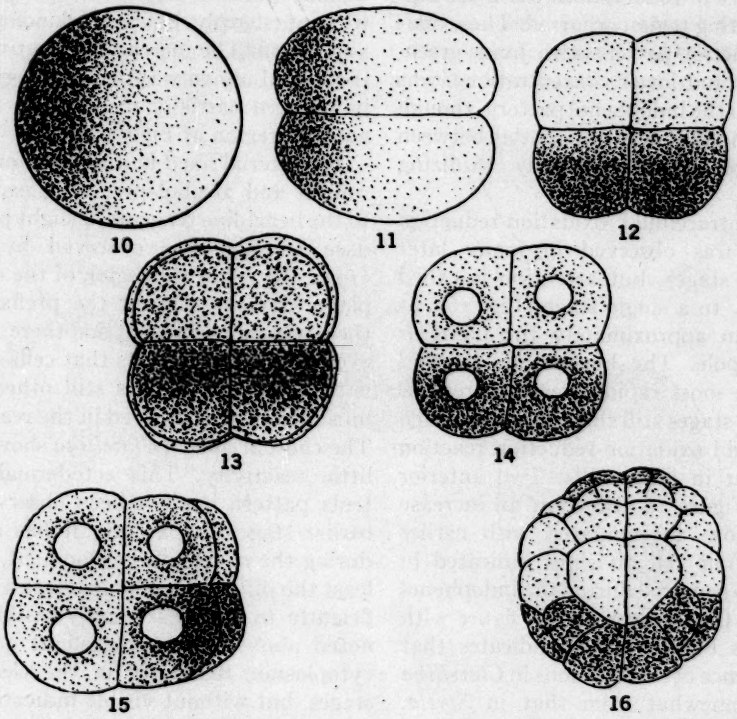
THE FERTILIZED EGG AND CLEAVAGE

Following fertilization, a new pattern appears. Its most conspicuous feature is a region of very rapid and intense indophenol reaction and primary oxidation of reduced dyes in a part of the undivided egg and during cleavage in certain blastomeres or parts of blastomeres. This is very different from the polar oöcyte pattern, and in *Clavellina* the part of the egg in which it appears is not visibly different from other parts before its appearance. It is evident from later developmental stages that the intensely reacting region is in the prospective posterior region of the egg. Figure 10 is a polar view of the reaction in the undivided fertilized egg. In earlier studies of several ascidian species by means of indicators, this pattern has appeared, with both the benzidine and the indophenol reaction (Ries, 1937, 1939; Reverberi and Pitotti, 1939). Moreover, it corresponds very closely in position with the localization of the natural yellow pigment in *Styela* (=*Cynthia;* Conklin, 1905). Rate and intensity of reaction decrease slightly in the anterior direction. Other parts of the egg cytoplasm show only slight and slow indophenol reaction with the dilute reagents and slight and slow oxidation of reduced dyes. In the hope of tracing more completely the development of this pattern, numerous attempts have been made during four seasons to fertilize eggs as desired, but without success, either by self-fertilization or otherwise. However, naturally fertilized eggs and all stages of cleavage were obtained in large numbers each season, and results as regards visible indicator patterns were consistent throughout.

In the two-cell stage the region of greatest reactivity is localized in the posterior part of each cell (Fig. 11), and in the four-cell stage in the two slightly smaller posterior cells (Fig. 12). With division into eight cells, the rapidly reacting region is localized in the two posterior blastomeres nearer the "vegetal" or basal polar region, as indicated in Figure 13. The boundaries of this region are not so sharply defined as those of the natural yellow pigment in *Styela*, as indicated by Conklin. The graded decrease in reaction appears, as in earlier stages, and there is also a decrease basipetally (Fig. 13). The two posterior apical blastomeres, sister-cells of the deeply reacting blastomeres, react more rapidly than do the four anterior cells (Fig. 13; also Fig. 15). Slow and slight reaction appears throughout

the cytoplasm of the four anterior cells. In the earlier cleavage stages a perinuclear zone of slightly more rapid reaction than in the cytoplasm generally appears in the blastomeres. In Figures 14 and 15 this zone is indicated by slightly

by Reverberi and Pitotti (1939). The essentially identical localization of the region of most rapid reaction with the yellow crescent formed by natural pigment in the fertilized egg and cleavage stages, as observed by Conklin in *Styela*,



FIGS. 10–16.—Fertilized undivided egg and cleavage stages. Nuclei omitted except in Figs. 14 and 15. Fig. 10, the rapid and intense reaction in the posterior region—the prospective mesoderm—of the undivided egg, slight reaction throughout the cytoplasm. Fig. 11, two-cell stage, polar view. Fig. 12, four-cell stage from apical pole. Fig. 13, eight-cell stage from posterior side. Figs. 14 and 15, combined optical section and general view of four- and eight-cell stages to show the perinuclear zone of slightly greater reactivity than the cytoplasm in general. Fig. 16, later cleavage from basal polar region, intense oxidative reaction in prospective mesoderm, slight elsewhere, but slightly greater in two anterior cells.

deeper shading than in the rest of the cytoplasm. In the two rapidly reacting blastomeres it is less clearly visible than in other cells but can still be seen in the living egg. This perinuclear zone appears to be indicated in some of the figures of the benzidine reaction (Ries, 1939, Fig. 1, *b*, *c*, *d*) but was apparently not noted

was noted in the earlier papers and is again pointed out here. Moreover, a perinuclear zone of yellow pigment is shown in Conklin's Figures 32–38, corresponding rather closely to the perinuclear zone of more rapid reaction in *Clavellina*, except that the zone of indophenol reaction and dye oxidation appears to be some-

what more extensive and less sharply bounded than the perinuclear zone of yellow pigment.

The same differentials in pattern appeared in indophenol reduction by hydrosulphite until toxic effect and retardation or absence of reduction in the most rapidly reacting region occurred. The oxidation-reduction pattern with Janus green, 1/10,000 and lower concentrations, was similar to the indophenol pattern, though with somewhat less differential between the more and less rapidly oxidizing regions.

The intracellular oxidation-reduction pattern was observed in many later cleavage stages, but attention is called here only to a single stage (Fig. 16), as seen from approximately the basal or vegetal pole. The blastomeres derived from the most rapidly reacting regions of earlier stages still show the same much more rapid oxidation-reduction reaction than that in other cells. Two anterior cells of Figure 16 also show an increase in reaction, as compared with earlier stages. Four cell divisions indicated in Figure 16 occurred during the indophenol reaction. Comparison of this figure with Conklin's Figures 42–45 indicates that the sequence of cell divisions in *Clavellina* differs somewhat from that in *Styela*, though the localization of the region of most rapid oxidation-reduction remains the same as that of the yellow pigment.

GASTRULA STAGES

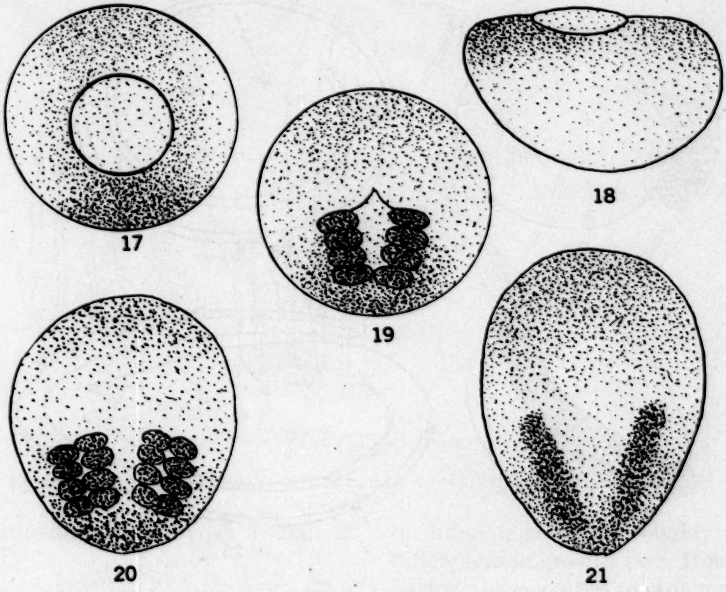
Large numbers of early and later gastrulae have been subjected to the indophenol reagents and dyes with definite and entirely consistent results, not only as regards the most rapidly reacting regions but in other parts. Only the gastrula ectoderm is shown in Figures 17 and 18. The anterior and posterior ectodermal regions both show more rapid oxidation-reduction than other parts. The posterior region, which will become the ectoderm of the larval tail, reacts more rapidly than does the anterior neural-plate region. The ectoderm lateral to the blastopore reacts less rapidly, and the reaction decreases ventrally. This ectodermal pattern is a gradient pattern without sharply defined boundaries in any region. The increase in reactivity in the neural region was perhaps beginning in the two partially visible cells in the anterior region of Figure 16.

In material fixed in a mixture of chloroform and alcohol preceding exposure to the benzidine reagents, a slight peroxidase reaction was observed by Ries (1939, Fig. 2) in the region of the neural plate, though, without the prefixation, there was no visible reaction there. However, his figure suggests that cells of the notochord and perhaps still other cells must have been involved in the reaction. The chordal cells in *Clavellina* show very little reactivity. This ectodermal gastrula pattern has not been observed in earlier stages; apparently it has arisen during the course of development, or at least the differentials have increased sufficiently to render it clearly visible. As noted above, there is a slight general cytoplasmic reactivity in the cleavage stages, but without visible indication of any regional differential. Perhaps further study with different concentrations of reagents and with attention particularly directed to this point might bring to light further slight differentials in earlier stages of the living embryos.

In somewhat later gastrulae the ectodermal pattern remains almost unchanged. The gradient decreasing anteriorly from the tip of the gradually elongating larval tail and posteriorly from the anterior end of the gastrula becomes slightly more distinct, and the rapidly reacting prospective muscle cells of the tail appear as a row of four cells on each

side of the median line (Fig. 19). These divide, producing two rows of four each (Fig. 20). The rows of four nearer the median line in Figure 20 show slightly less reaction than do the cells of the more lateral row. According to Conklin, mesenchyme cells of *Styela* are less deeply colored by the natural yellow pigment (e.g., Conklin's Figs. 47 and 48) than are mesoderm cells. The benzidine reaction the mesoderm cells—in consequence of differential toxic effect.

As the gastrula begins to elongate posteriorly in early stages of larval tail formation, the ectodermal pattern in the tail region becomes more distinct and the gradient more marked (Figs. 20 and 21). The mesoderm cells continue to divide, the two rows elongate, and the intense reactivity remains the same. The exact



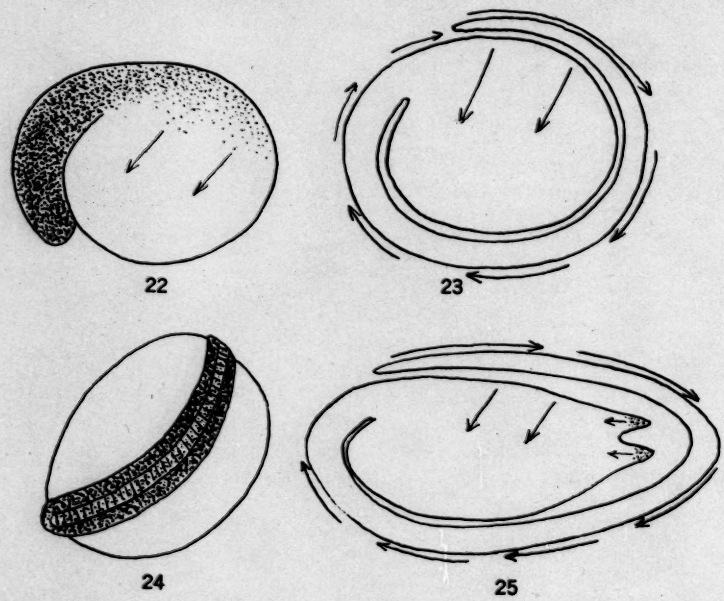
FIGS. 17–21.—Gastrula stages. Figs. 17 and 18, ectodermal pattern in early gastrulae. Figs. 19–21, pattern of later gastrulae and beginning elongation of larval tail.

also indicates somewhat similar differences (Ries, 1939, p. 103). The fate of the cells of the less rapidly reacting rows in *Clavellina* has not been followed. They are perhaps prospective mesenchyme cells; but, if they are, the cell divisions by which they arise differ from those in *Styela*. Intracellular oxidation and reduction of dyes as well as the indophenol reaction indicate the same gastrular pattern until reduction is retarded or fails to occur in the most susceptible cells—numbers of cells in each row become difficult to determine, and in Figure 21 individual cells are not indicated.

LARVAL DEVELOPMENT

As elongation of the larval tail progresses, the ectodermal reactivity increases further; but, as the mesoderm cells differentiate, their reactivity apparently decreases somewhat. In Figures 22 and 23 the gradient pattern of the tail is indicated without distinction between

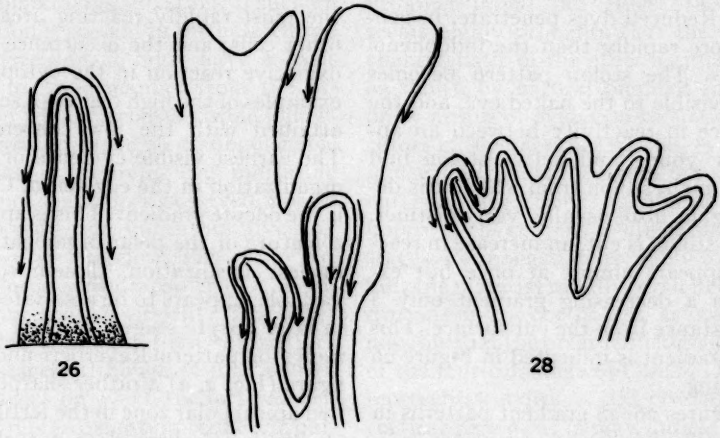
ectoderm and mesoderm. The caudal gradient decreases basipetally from the tail tip and in the body from the dorsal region ventripetally. The arrows in Figures 22, 23, and 25 point in the direction of decreasing rates of reaction. However, secondary reduction of intracellular indophenol and oxidized methylene blue often occurs over a greater distance basipetally elsewhere. Figure 24 indicates the difference in rate of intracellular oxidation of Janus green in cells of the notochord and other parts of the larval tail. The indophenol differential is similar. In Figure 25—the latest stage used as indicator material—the gradient pattern is essentially like that of earlier stages, except that the developing papillae at the an-



FIGS. 22–25.—Stages of larval development, gradient pattern indicated by shading and arrows. Fig. 23, difference in reaction in notochord and rest of tail.

from the tail tip without external oxygen decrease. Apparently, activity is so great in these regions that the oxygen available within the membrane is not sufficient to keep indophenol and methylene blue oxidized. Janus green, as might be expected, usually remains oxidized in this region unless hydrosulphite is used. In consequence of these secondary reductions in the terminal tail regions, the depth of indophenol reaction and of primary dye oxidation may gradually become deeper in the basal regions than terior end show a distinct gradient, decreasing basipetally. With high concentrations of intracellular indophenol or oxidized dyes, reduction may be retarded or fail to occur over a variable distance from the tail tip, the same region which often undergoes secondary reduction without external oxygen decrease preceding this toxic effect. Following the basipetal retardation or absence of reduction, cytolysis may begin at the tail tip and progress basipetally. In short, there is a basipetally decreasing susceptibility

to the indicators in the tail. Entodermal patterns in these early larval stages were not specially investigated. However, entodermal reactivity was slight in earlier stages, and no marked increase was indicated in these larval stages. Ries (1939, pp. 114–16) found that during the metamorphosis of *Ascidiella* the benzidine reaction in the mesenchyme, the ferential toxic effect, also decreasing basipetally. The stolons differ in reactivity, apparently with differences in growth rates. In the tunic the cells are scattered and vary greatly in numbers. In almost all stolons observed, numbers of tunic cells in a given area decrease basipetally from a more or less extensive region at the tip. The gradient of decrease varies



FIGS. 26–28.—Stolons. Gradient pattern indicated by arrows, except the injury pattern in Fig. 26

entoderm, and the nervous system increased.

STOLON PATTERNS

Indicator patterns of ascidian development from stolons have not been investigated, but a few data concerning growth patterns of *Clavellina* stolons are noted here. A stolon gradient, decreasing basipetally from the tip, has appeared with the indophenol reaction and with intracellular oxidation of dyes reduced by hydrosulphite in every case of a large number examined. The reduction gradient of indophenol and of oxidized dyes also decreases basipetally, and the reoxidation gradient is like that of primary oxidation until these are altered by different stolons, probably also with differences in growth rate. However, the basipetal gradient in the tunic cells is not merely a matter of the decrease in numbers of cells. It is clearly evident from a comparison of different stolon levels that indophenol reaction and dye oxidation decrease in rate basipetally from the stolon tip, i.e., the reactivity of the cells decreases basipetally.

The inner tissue of the stolon consists of a thinner outer layer and an inner mass. This may extend almost to the extreme stolon tip (Fig. 26), or its apical end may be at varying distances from the tip, perhaps in some cases because of retraction (Fig. 27). The gradient pattern in the tunic becomes visible earlier than

that in the inner layers, and in the latter the pattern appears earlier in the outer than in the inner tissues. The difference between tunic and inner tissues is greater with indophenol than with intracellular oxidation of dyes and may be largely or entirely a result of differences in rates of penetration. Primary dye oxidation in solutions reduced by hydrosulphite is much more rapid than the indophenol reaction. Reduced dyes penetrate, in general, more rapidly than the indophenol reagents. The stolon pattern becomes clearly visible to the naked eye, and the difference in reactivity between an apparently young and active stolon bud and the main stolon from which it is developing is also usually very distinct. When a stolon is cut, an increase in reactivity appears almost at once but extends in a decreasing gradient only a short distance from the cut surface. This injury gradient is indicated in Figure 26 by shading.

In Figures 26–28 gradient patterns in three different forms of stolons are indicated by arrows, except the injury gradient in Figure 26. Figure 26 appears to be a rapidly growing stolon; in Figure 27 the inner tissues appear to have undergone retraction; Figure 28 has five short, apparently secondary, branches budding from two main branches. In the secondary branches the gradients are very distinct (indicated in only one but present and similar in all). In the lower regions of the stolon the differentials are less marked.

DISCUSSION

Comparison of the data of this paper with those of Ries (1939) and Reverberi and Pitotti (1939) seems to indicate that use of the indophenol reagents in very low concentrations and also of low dye concentrations reduced by the nontoxic sodium hydrosulphite brings to light some slight regional differentials not recorded in the earlier studies. Moreover, the possibility of repeated reduction and reoxidation provides a means of making visible regional differential susceptibilities to certain degrees of toxic effect of the indicators. The distinct polar gradient in the growing oöcyte from very early stages; the regional differentials in the indophenol reaction and dye oxidation, not only in the most rapidly reacting areas but in other cells; and the occurrence of slight oxidative reaction in the cytoplasm are examples of the high degree of sensitivity attained with the low concentrations. The earliest visible evidence of regional organization in the egg cell of *Clavellina* is the oöcyte gradient. This is apparently a feature of the polar organization; and nuclear localization, though somewhat variable, appears to be associated with it in later oöcyte stages. In their study of indicator pattern Reverberi and Pitotti figure (Fig. 2, *a*) a rather sharply delimited apical polar zone in the fertilized egg of *Phallusia* which does not show the indophenol reaction. Conklin (1905, Figs. 1 and 2) observed a similar apical zone, differing in color from the rest of the egg at the time of polar body formation in *Styela*. According to Ries (1937, Fig. 10, *a*) the unfertilized egg of *Ciona intestinalis* from the oviduct shows the same organization as the fertilized egg, i.e., a dorsal, rapidly reacting crescent corresponding to the yellow-pigmented crescent in Conklin's material, though in that case it becomes visible only after the redistribution of cytoplasm associated with fertilization. Reverberi and Pitotti have found no such organization in the unfertilized eggs of the three species studied by them. In *Clavellina* there is no visible evidence of such organization preceding fertilization. The case illustrated by Ries appears to be one in which the organization characteristic of the fertilized egg

has been observed in an egg regarded as unfertilized. Either the egg of *Ciona* differs greatly from *Styela* and other ascidian eggs thus far studied by means of indicators, or the egg illustrated by Ries has been fertilized.

Extensive experiments on merogony have led Dalcq (1932 and earlier reports of this work) to the conclusion that the unfertilized egg of *Ascidiella aspersa* possesses a bilateral organization. If such an organization is present in the unfertilized egg of *Clavellina*, it must have a basis differing in some manner from the organization which becomes visible following fertilization, since no distinguishable differentials in the living egg, indicating its existence, have been observed, nor do oxidation-reduction indicators give any evidence of its presence. This, however, does not necessarily mean that it is not present but that, if present, it is either different in character from the pattern of later stages, although it may constitute the basis on which the latter develops, or, if it is an oxidation-reduction pattern, the regional differentials are so slight preceding fertilization that the indicators fail to make them visible.

Some thirty years ago the writer attempted to determine whether intracellular reduction of potassium permanganate could be used as an indicator of at least some of the physiological differentials concerned in gradient pattern. Among the organisms used as material were developmental stages of the ascidian, *Corella willmeriana*, a form abundant in Puget Sound. The results of this work with permanganate on the ascidian material appeared some years later (Child, 1927). Pages 475–81 and Figures 11–23 of that paper give the results obtained with permanganate. In early cleavage, only a polar gradient decreasing basipetally was observed; but in gastrula and larval stages the pattern was almost the same as in *Clavellina*, except that in the gastrula the ectoderm of the neural region showed increase in rate of reduction slightly earlier than did the prospective caudal region, as indicated in Figures 12–14 of the *Corella* paper for the gastrula and Figures 15–19 for early larval stages. The high reactivity of the prospective mesoderm did not appear in earlier stages but was apparently visible in the developing larval tail (Figs. 16–19). It seems highly probably that the pattern characteristic of the other ascidian eggs, as shown by natural pigment in *Styela* or by means of oxidation-reduction indicators in several other species, is present in *Corella* from early stages on. At present, only suggestion is possible as regards failure of the pattern to appear in earlier stages. Permanganate is highly toxic and kills the material rapidly, even in concentrations of 1/5,000 or less, as used. It is possible that the polar reduction gradient of the four-cell stage of *Corella* (Fig. 11) represents conditions still present in the cortical region, or it may indicate a differential in yolk distribution. There is also the possibility that the highly susceptible mesodermal region is killed and the enzymes destroyed almost at once by the permanganate. The gastrular pattern observed with permanganate concerns only ectoderm, but the gradient of the larval tail seems to involve mesoderm as well as ectoderm, though ectoderm may still be the chief factor in it. One further point of interest is that, as the tail approaches the stage of resorption, the gradient disappears entirely or almost entirely in *Corella;* reduction of permanganate becomes much less rapid and occurs only in scattered spots (Figs. 20 and 21 of the *Corella* paper). However, the similarities between the ectodermal permanganate pattern in *Corella* and the indophenol and dye oxidation and reduction pattern in *Clavellina* are of interest.

Another point of interest as regards the ascidian indicator patterns is that the regions of more rapid reaction, even the prospective mesodermal region, are not sharply delimited in early stages but grade into less rapidly reacting regions. The yellow pigment of *Styela* appears in Conklin's figures to be more sharply delimited than the indicator pattern. The gradations at the borders of the regions of high reactivity and the occurrence of slight indophenol reaction and dye oxidation in the cytoplasm generally suggest that the ascidian pattern is not, in the strict sense, a pattern of purely qualitative regional differences, at least during earlier developmental stages. It appears to be in some degree a pattern of quantitative differentials. Moreover, as development progresses, changes in enzyme activity occur, e.g., in the gastrula ectoderm. In certain cells or cell groups enzyme activity increases, and, as the muscle cells differentiate, their reactivity decreases somewhat. Ries (1939) has noted that the benzidine reaction in mesenchyme and entoderm increases during metamorphosis.

SUMMARY

1. This paper is a study of intracellular developmental pattern in the ascidian *Clavellina huntsmani* by means of the indophenol reaction, intracellular oxidation of methylene blue and Janus green reduced by sodium hydrosulphite, reduction of intracellular indophenol and oxidized dyes by hydrosulphite, and reoxidation of these indicators following reduction. The indophenol reagents were used in very much lower concentrations than in the Nadi reaction, so that the intracellular reaction and intracellular oxidation and reduction of dyes occur while the material is still living.

2. From very early stages there is a gradient in the growing oöcyte, decreasing from the region of the cell farthest from the "stalk." The nuclear position is somewhat variable, but, at least in later oöcyte stages and in full-grown egg cells, the nucleus is usually in or near the region of greatest reactivity.

3. The most conspicuous feature of the pattern following fertilization is like that described in earlier work with indicators —a very great reactivity in the posterior region of the egg, the prospective mesoderm. This is almost identical with the posterior zone of natural yellow pigment in *Cynthia* (=*Styela*). This region can be followed in certain cells by its reactivity through cleavage and gastrula stages and to its differentiation chiefly into muscle cells of the larval tail.

4. In the eight-cell stage and later, the two apical posterior cells—sister-cells of the two basal cells containing the region of very high reactivity—show somewhat less reactivity than do the anterior cells. A perinuclear zone of greater reactivity than the rest of the cytoplasm appears in the earlier cleavages, apparently somewhat more extensive than a perinuclear zone of yellow pigment in *Styela*. There is also slight reactivity in the cytoplasm of all cells.

5. In the early gastrula before elongation of the tail begins, reactivity increases in the prospective tail ectoderm and to a lesser degree in the neural plate and still less in regions lateral to the blastopore. The intensely reacting mesoderm cells appear in longitudinal rows, increasing in length by continued division.

6. With beginning elongation of the tail, further increase in reactivity in the tail ectoderm is indicated, with a gradient decreasing basipetally. A region varying in length at the tip of the growing tail may secondarily reduce indophenol and oxidized dyes without external oxygen decrease. With differentiation

of the muscles of the larval tail, the reactivity decreases somewhat.

7. In the larval body reactivity decreases from the dorsal region. In the developing papillae there is marked increase in reactivity, with a gradient decreasing from their tips.

8. With increase in intracellular concentration of indophenol and oxidized dyes, toxic effect becomes evident in differential retardation or absence of reduction. Susceptibility to toxic effect decreases with decrease in original reactivity, with a resulting greater or less reversal of pattern differentials.

9. The absence of sharp boundaries in the regions of differing reactivity indicates that the pattern of *Clavellina* is in some degree quantitative rather than strictly a pattern of qualitative regional differences.

10. In all of many living stolons subjected to indicators, an oxidation-reduction gradient has appeared, decreasing basipetally from the tips, both in the tunic and in the inner tissues. The reactivity apparently varies with growth activity. When a stolon is cut, an injury gradient, extending a short distance from the cut end, appears almost at once.

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ON THE SECRETION AND EXCRETION BY THE HEPATIC CELL OF ALBINO MICE[1]

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THE difficulties which are met in investigations on the secretion and excretion of the hepatic cell are perhaps even greater than those of investigations on the formation of glycogen. A point which has caused profound confusion in the literature is the fact that these two processes are not separated from each other and therefore not easy to analyze. According to one theory, bile secretion should actually be an excretion because catabolic products, such as bile pigments and bilirubin, probably produced by the entire reticulo-endothelial system and transported by the blood, become extruded into the bile capillaries. However, when comparing the chemical structure of blood and bile bilirubin, it becomes obvious that bilirubin is changed

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in the hepatic cell. Some investigators (see Pfuhl, 1932) deny any active process of the hepatic cell with regard to the extrusion of bilirubin. According to them, the cells of Kupffer (the stellate cells) transport these substances directly from the blood toward the bile capillaries. Since these cells show no distinct contact with the bile capillaries, this assumption can be considered erroneous.

Although the chemical structure of bilirubin is changed to some extent by the hepatic cell, its extrusion must be considered an excretion because no drastic alterations occur, leading to the formation of an entirely new substance. Bile acids, however, are produced by the cell (from cholesterol?), so the extrusion of these bile components is a true secretion.

The excretion processes themselves are still rather obscure, despite the many attempts made to analyze them. It is generally held that the excretions are first accumulated by the cells of Kupffer, after which they are transferred to the hepatic cells, which extrude them into the bile capillaries. The hepatic cell would therefore be only a (passive) passage. Other investigators (e.g., Lepehne, cited by Pfuhl, 1932) consider the excretion to be an active hepato-cellular process. Corpuscular bodies remain in the reticulo-endothelial cells and are therefore never found in the hepatic cells.

Because secretion and excretion are not easily distinguished from each other and because the ultimate products of both processes are extruded into the same bile capillaries, it is small wonder that confusion prevails, particularly concerning the question of which cell structures co-operate (morphologically) with these processes. The old theory that the nucleus produces bile (Baum, 1884; Browicz, 1905) has been abandoned. Policard (1909*a*, *b*) observed a granular disintegration in the hepatic cells of frogs and dogs during digestion. He believed that this was the first step in intracellular bile formation. Berg (1920) demonstrated a distinct swelling of the mitochondria of the liver of the newt after feeding with cholagogenic substances. This swelling would be morphologically linked with bile formation. Cramer and Ludford (1926/27) studied the changes in the Golgi "apparatus" and found that it broke up into fragments which moved toward the bile capillaries and poured out (!) their contents into them. Nassonov (1926) believed that he could demonstrate a morphological relation between the Golgi substance and the dye granules which appear after the injection of acid dyes into the blood. He concluded that these granules (and other excretions) must originate in the "strands" of the Golgi substance and that after some time they would be extruded into the bile capillaries.

Because an origin *de novo* in the undifferentiated cytoplasm of secretions and excretions is unknown, they would be formed or accumulated, according to existing theories and conceptions, either in the mitochondria or in the Golgi substance. The latter substance would even act somewhat as a canalicular "apparatus" (Cramer and Ludford, 1926/27). However, as it is now known that the Golgi substance is not an "apparatus," but very probably an alloplasmatic substance, and as it has been demonstrated before that the mitochondria play a direct part in the formation of glycogen (van Weel, 1941), it seemed interesting to investigate the problems of hepatic secretion and excretion once more.

MATERIAL AND METHODS

Albino mice were killed, by bleeding, at regular intervals of 3 hours over a

period of 24 hours. A small part of the liver was fixed in Champy's fluid and a part of this material, thus fixed, was osmicated at 37° C. for 10 days to demonstrate the Golgi substance. Other portions of the same liver were fixed in formol 1:10 and barium chloride. This material was stained with acid-fuchsin at pH 2 after Forsgrenn (bile staining). The Champy-fixed material which was not osmicated was stained after Heidenhain. For general purposes, parts of the livers were fixed in Bouin's fluid and stained either with iron haematoxylin or in hemalum-eosin.

For the experiments on excretion, mice were injected (intraperitoneally or subcutaneously) with 1 cc. of 0.6 per cent of trypan blue solution (in sterile Ringer's solution). The injections were repeated every 24 hours for 1–6 days. The liver was quickly removed ½ hour after the last injection; most of it was fixed in Bouin's fluid or in "Susa" (4 cc. glacial acetic acid + 4.5 gm. corrosive sublimate + 2 gm. trichloroacetic acid + 80 cc. distilled water + 0.5 gm. sodium chloride), and the remainder was used for supravital examination. The fixed material was counterstained with Bismarck brown or carmine. Fixation of vitally stained tissue in formol or alcoholic solutions like Carnoy's solution did not produce reliable results. Tiny granules tend to disappear, and the larger ones often show marked dislocations, as comparison with the living tissue reveals.

THE SECRETION PROCESSES

The liver of albino mice shows a distinct 24-hour cycle; it contains a maximum of glycogen in the early hours of the day and a minimum between 5:00 and 8:00 P.M. (van Weel, 1941). According to Forsgrenn (1928–30), the bile contents of the liver show the reverse cycle: the liver would display a minimum of bile when a maximum of glycogen occurs. This could be confirmed completely. The bile granules as demonstrated with Forsgrenn's method appear scattered through the cell. There is no accumulation or concentration of bile in, at the surface of, or near any cell-organoid or structure. Even during the period of increased bile production (between 11:00 A.M. and 5:00 P.M.), no special localization of bile granules was ever observed. They appear as small, bright-red granules. There are few granules in the minimum period and many densely packed ones in the maximum period, but they always appear evenly scattered. However, the fixation employed does not lend itself to delicate cytological purposes. It is therefore impossible to draw any definite conclusion concerning the morphological relation between bile granules and certain cell structures from the pictures obtained by this method.

1. THE NUCLEUS

No morphological or chemical changes of the nucleus related to the cyclic changes in the hepatic cells were observed. Certainly, neither a nucleolar extrusion (the karyoblast-extrusion of Baum, 1884) nor a degeneration of the nucleus occurs. Neither delicate canals running from the nucleus toward bile capillaries (Browicz, 1905) nor a chromidial extrusion was ever seen. There is, therefore, no direct morphological relation between the nucleus and bile formation.

2. THE MITOCHONDRIA

The mitochondria show a distinct metamorphosis during the 24-hour cycle, changing from delicate filamentous structures in the glycogen-free cell into thick, plump rodlets and granules which concentrate glycogen in increasing quantities until they are transformed into

glycogen "droplets." Only a few mitochondria remain filamentous during the cycle. When glycogen is mobilized by the liver, the glycogen "droplets" disappear totally, and the few remaining mitochondria break up into fragments, which grow out to new mitochondrial filaments and ultimately become transformed again into the glycogen "droplets" (van Weel, 1941). In the period in which filamentous mitochondria occur in the small, isolated fragments. They often seem branched, sometimes even net-shaped. They often occur near the nucleus (never completely encircling it as a "belt"), but sometimes they can also be found near the bile capillaries. These capillaries are practically all closed at this stage (Fig. 1, *a*) and are certainly not wrapped in osmiophilic substances. This is easily demonstrated in tangentially sectioned cells (Fig. 1, *b*). The os-

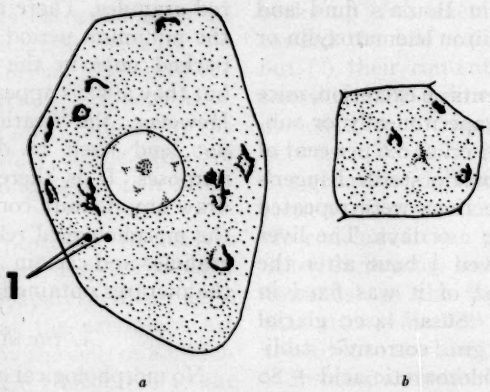

FIG. 1.—Golgi substance in the storing cell. *a*, transverse section through a cell; *b*, superficial section through a cell; *l*, lipochondria.

cell (5:00–8:00 P.M.), granular swellings regularly become visible in these structures.

3. THE GOLGI COMPLEX

The Golgi complex seems to be an extremely variable structure in the hepatic cell. It is therefore impossible to find a single, well-defined, typical form or shape, as cells in the same phase of activity may show quite different osmiophilic structures. However, by studying many sections, one may find the following shapes as more or less average or "typical."

In the so-called "storing" cell (maximal contents of glycogen) the Golgi complex appears in the form of relatively

miophilic fragments are always separated from one another and never tend to fuse.

During the early period of increase of bile contents, the Golgi complex tends to move toward the bile capillaries, and the Golgi substance becomes accumulated in these areas. In transverse sections of the cell one often gets the impression of a relatively small quantity of Golgi substance present (Fig. 2, *a*). However, a top view of the cell indicates the contrary (Fig. 2, *b*). The osmiophilic substance "tracks" the bile capillaries rather accurately. Near the blood sinusoids, on the other hand, practically no Golgi substance seems to occur.

Soon the fragments seem to break up into series of tiny granules, which

gradually disappear. Accordingly, the "empty" (glycogen-free) hepatic cell is practically totally deprived of Golgi substance. In this phase the bile capillaries generally become brilliantly distinct. Sometimes they even show a delicate, impregnated "wall." Because the bile capillaries do not possess a true wall

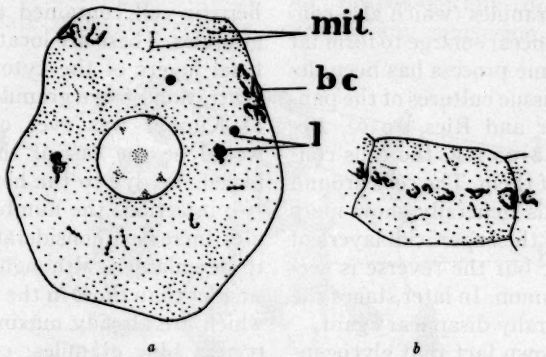

FIG. 2.—Golgi substance during first hours of bile formation. *a*, transverse section through the cell; *b*, view on top of the cell; *bc*, bile capillary; *l*, lipochondria; *mit*, mitochondria.

of their own but are formed by a thin modified layer of dense cytoplasm of the hepatic cell (Clara, 1930, 1931), the impregnated "mass" cannot be the Golgi substance of the bile capillaries themselves but probably represents the last remnants of the Golgi substance of the hepatic cell. This fits well with the fact that a top view of the cell never displays totally blackened capillaries, but only small parts of them appear black. These parts seem to correspond with the localization of the former Golgi fragments.

Between 8:00 and 11:00 P.M. new Golgi substance appears near the nucleus (Fig. 3, *a*). How it originates could not be ascertained. It apparently appears in close touch with a comparatively small number of lipochondria (Fig. 3, *a*, *b;* see also van Weel, 1941). These granules very probably contain fatty acids and lipid substances between 5:00 and 8:00 P.M., which disappear when the Golgi substance develops. They then appear wrapped in a layer of well-impregnated, but not yet extended, osmiophilic substance (Fig. 3, *b*). But even in stages of well-developed Golgi material the (morphologic) relation between lipochondria and the osmiophilic substance is often obvious (Fig. 1, *a*). Therefore, it seems highly probable that the Golgi

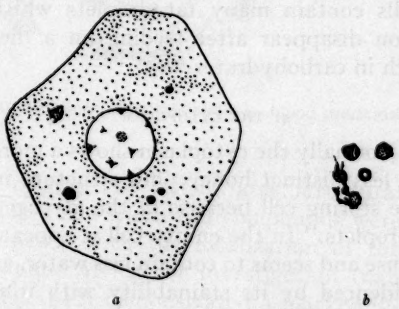

FIG. 3.—Development of the Golgi substance. *a*, transverse section; *b*, lipochondria and different stages of development of Golgi substance.

substance is in some way "produced" by the lipochondria (compare also with Ries, 1935; van Weel, 1937, 1938 *a*, *b*, 1939).

During the reduction of Golgi sub-

stance, two interesting features can be observed. First, the granular swellings of the filamentous mitochondria seem more or less osmiophilic (Fig. 2, *a*); second, small fat droplets often appear. How the latter ones arrive could not be established exactly. However, as the lipochondria often show constrictions in this phase, it is not impossible that their daughter-granules (which also contain fatty substances) enlarge to form fat droplets (this same process has been described, e.g., in tissue cultures of the pancreas by Fischer and Ries, 1936). Between 2:00 and 8:00 P.M. the cells contain only a few of them. The cells around the vena centralis generally show more fat than those of the superficial layers of the liver lobules, but the reverse is certainly not uncommon. In later stages the fat droplets generally disappear again.

It is a well-known fact that glycogen-free cells sometimes contain much fat. This is very clearly demonstrated in the liver of starved mice, which contains only a very small amount of glycogen (van Weel, 1941). Although this liver is certainly not a fatty one *senso stricto*, the cells contain many fat droplets which soon disappear after feeding on a diet rich in carbohydrates.

4. THE CYTOPLASM

Normally the cytoplasm shows a more or less distinct honeycomb structure in the storing cell because of the glycogen "droplets." In the empty cell it appears dense and seems to contain less water, as evidenced by its stainability with iron haematoxylin.

THE EXCRETION

Because it is practically impossible to examine the excretion of bilirubin, the extrusion of the likewise negatively charged, but semicolloidal, trypan blue was studied to determine whether the excretion of this substance takes the same course as the normal bile secretion. Contrary to the assumption that trypan blue is exclusively taken up by the stellate cells, which then pass their load on to the hepatic cell, as opposed to the fact that the latter are unable to take up the dye themselves from the blood, it was obvious that after only one injection the hepatic cell contained a few tiny blue granules. These are located in the superficial layers of the cytoplasm. No concentration of dye granules near the cells of Kupffer was ever observed, which would be the case if these cells transferred the dye to the hepatic cell. After two injections the number of blue granules increased considerably. They seem to be enlarged, although they are much smaller than those in the cells of Kupffer, which are already maximally filled with trypan blue granules; even their cytoplasmic protrusions contain dye. In this stage of excretion the blue granules still appear irregularly scattered through the hepatic cell, but now they are found in the center of the cell as well as in its superficial layers.

After three injections the picture of the cell differs markedly from the former two. *A distinct concentration of blue granules now occurs around the nucleus.* This concentration generally shows a dense accumulation of dye granules toward the side of the bile capillaries. This accumulation is much more evident after four injections, and now the granules seem to "flow" from the nuclear concentration toward the bile capillaries (Fig. 4). The closer this "flow" approaches the capillaries, the less dense it becomes. This typical distribution of trypan blue can be observed readily in fixed and in living tissue. Granules scattered through the cell between blood sinusoids and the nuclear accumulation appear in addition to those of the "flow."

When the administration of dye is discontinued, the general picture does not change markedly until 12–14 days after the last injection. By this time the nuclear accumulation diminishes very much, and the "flow" of granules toward the bile capillaries becomes less dense and therefore less distinct. In the outer layers of the cell a few blue granules still occur. The stellate cells still contain

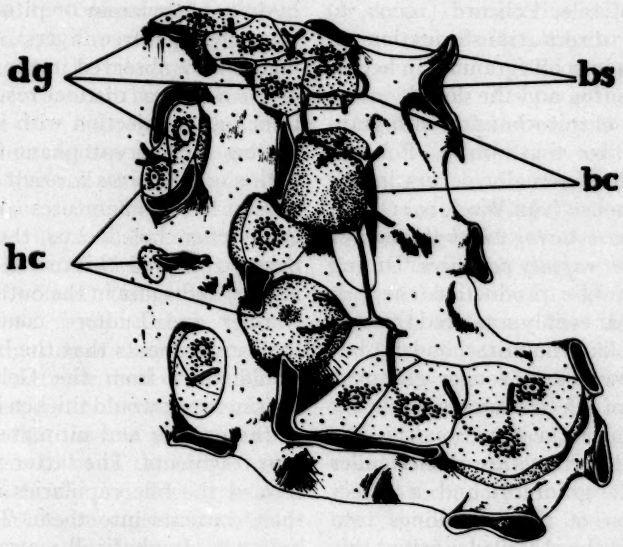

FIG. 4.—Excretion of trypan blue, semischematic reconstruction. *bc*, bile capillaries; *bs*, blood sinusoids; *dg*, dye granules; *hc*, hepatic cells.

many large dye granules. A month after the last injection, the hepatic cells are practically deprived of trypan blue, and the cells of Kupffer rarely contain blue granules at all. After 2 months no trypan blue could be demonstrated in the liver.

CONCLUSIONS

The microscopic observations concerning the quantity of bile granules in the hepatic cells during the 24-hour cycle reveal a distinct sequence of bile production: a minimum occurs when the hepatic cell contains a maximum of glycogen (2:00–8:00 A.M.), a maximum from 5:00 to 8:00 P.M., when the cell contains hardly any glycogen. The bile granules appear scattered throughout the cell. Apparently, no definite concentration of bile occurs in the hepatic cell. Because the fixation used for demonstrating the bile microscopically does not permit drawing any conclusion on the mode of its origin, the cyclic and structural changes in various cell compounds were compared with the localization of the granules. An earlier investigation (van Weel, 1941) demonstrated a marked increase of mitochondria in the period of increased bile formation. It seemed natural to correlate both processes, despite the fact that the greater part of the mitochondria transforms into glycogen "droplets." The possibility remains that part of the mitochondrial substances changes into, or is morphologically related to, the

bile granules. The literature offers practically no support to such a relation between mitochondria and bile formation. Champy (1911) described a mitochondrial accumulation near the bile capillaries, but this has never been confirmed. Fiessinger and Lyon Caen (1910) only discussed the possibility of mitochondria producing bile (and fat). Lahouse and Langley (1910; cited by Pfuhl, 1932) consider the "cytoplasmic granules" to be the source of bile. Policard (1909*a*, *b*) described a direct transformation of mitochondria into bile granules in hepatic cells of the frog and the dog.

A swelling of mitochondria into granules similar to that which Policard (1909) describes actually occurs in the liver of the mouse (van Weel, 1941), but *only in the phase during which the number of bile granules rapidly decreases*. During the phase of bile production the bile granules appear evenly scattered through the cell, just like the mitochondria; but the latter now are no longer granular. The number of bile granules is markedly fewer than that of the mitochondria. The mitochondrial swellings are much smaller than the bile granules, and a direct transformation of these swellings into bile could never be observed, nor was this the case with any other mitochondrial substance. It seems, therefore, highly improbable that mitochondria or mitochondrial substances turn (directly) into bile, an assumption which stands in good agreement with the present general conception of bile formation.

The nucleus may be excluded from any direct bile production. Typical morphological changes do not occur. The fact that the bile granules seem to originate throughout the cytoplasm and that they do not appear first near the nucleus supports this assumption. A nuclear extrusion (Baum, 1884–86; Kremer, 1932) or the formation of bile-transporting canalicules (Browicz, 1905) was never observed in the hepatic cell of the mouse.

Cramer and Ludford (1926/27) have performed perhaps the most thorough investigations concerning the relation between Golgi substance and bile formation. They experimented with mice, rats, and guinea pigs. In normal animals they nearly always found a network or some isolated fragments near the nucleus. After administration of β-tetrahydronaphthylamin or pituitrin (to obtain glycogen-free livers), Golgi fragments were observed in the outer layers of the cell. Less distinct results were obtained after injection with insulin, after feeding with tryptophane-free food or with thyroid, or as a result of the influence of low temperatures. After ligating the ductus choledochus, the Golgi fragments appeared thickened, often canalicular, and again in the outer cell layers. Cramer and Ludford concluded from their experiments that the bile secretion would start from the Golgi network. This network would thicken in the course of the process and ultimately break up into fragments. The latter would move toward the bile capillaries and extrude their contents into them. The authors, however, emphatically stress the fact that the Golgi "apparatus" is very variable in shape in all stages of cell activity.

Bowen (1926) described a close relation between Golgi substance and certain "fuchsinophilic secretion granules" in the liver of the cat. Of the chemical nature of these granules, however, nothing was mentioned. Nassonov (1926), Makarov (1926), and Ludford (1928) always found a certain relation between Golgi substance and bile capillaries. Because all authors mentioned were convinced that the Golgi complex, as an *apparatus*, builds granular secretion, the same process would presumably also occur here. The localization of the Golgi sub-

stance in the cell would confirm this assumption.

In the present investigation no definite "network" could be found in any of the phases of the 24-hour cycle, neither laterally of the nucleus (Cramer and Ludford, 1926/27) nor around it (Cramer and Ludford; Nassonov, 1926) nor between the nucleus and the bile capillaries (Makarov, 1933; Ludford, 1928). The Golgi substance consists of fragments (also described by Cramer and Ludford) scattered through the cell (Figs. 1, 2, and 3), often near the bile capillaries but equally frequently near the nucleus. The sections give the impression that the Golgi substance is less conspicuous in those parts of the cell which are bounded by the blood sinusoids. During increased bile formation the Golgi fragments move toward the bile capillaries (compare with Cramer and Ludford), *but during this period they become greatly reduced.* A swelling or the formation of osmiophilic canalicules, which extrude their contents into the capillaries, certainly does not occur. It is obvious that most of the Golgi substance happens to be in the cell when it contains practically no bile granules. There was nothing to suggest that Golgi material was converted into bile, which is rather improbable because the bile granules appear scattered throughout the cell and certainly are not only where the Golgi substance happens to be.

Whether the Golgi substance plays any part in the extrusion of bile when it lies near the capillaries could not be ascertained. Because the cells contain no osmiophilic substance in the period of 5:00–8:00 P.M.(they now contain very much bile), the presence of the Golgi substance seems unnecessary for this process.

Likewise the lipochondria cannot be considered to produce the secretion material. Because lipochondria occur only in a small quantity, bile granules would be expected to appear in clusters in certain areas of the cell in the early stages of bile formation if they originated from the lipochondria by means of division. This, however, is not the case.

Considering all these facts, we are forced to conclude that the bile granules appear *de novo* in the cytoplasm. A question that remains open to discussion is whether the secretion appears in granular shape. In the living cells a relatively large number of granules are not present between 5:00 and 8:00 P.M. The cytoplasm during this period appears more or less dense and homogeneous (van Weel, 1941). Because bile has a different refractive index than cytoplasm (as control experiments revealed), it is not likely that bile granules would have a refractive index similar to cytoplasm and thus be invisible in the living cell. It is more plausible to assume that the secretion is formed and extruded in submicroscopic dimensions (as seems to be the case in the secretion cells of the crypts of Lieberkühn [van Weel, 1939]). Fixation could cause a disintegration of the cytoplasm, secondarily followed by a granular "concentration" (this often occurs also in living cells). According to this assumption, the bile granules would be artifacts, which, however, can give a decisive answer concerning the gross distribution of the secretion in the cell.

The investigations of Nassonov (1926) and Ludford (1926–28) and co-workers have demonstrated that the hepatic cell can take up semicolloidal substances, such as trypan blue. However, it still remains an open question whether the cells take up this dye out of the blood themselves or whether they obtain it from the stellate cells. According to Radt (1930), such substances are transferred to the hepatic cell exclusively by the latter cells. Experiments of Pfuhl (1932) on the

rabbit, on the other hand, showed that the hepatic cells contained more trypan blue than did the cells of Kupffer after subcutaneous injection of the dye, but less after intravenous injection. In the latter case a great part of the dye becomes precipitated and afterward phagocytized by the reticulo-endothelial system. In the former the blood contains practically only dissolved dye, which is readily taken up by the hepatic cells. In the rabbit an active resorption by the hepatic cells seems, therefore, an established fact.

In the liver of the mouse the same mechanism seems to exist. After one injection the hepatic cells contain a few trypan blue granules, although distinctly fewer than the cells of Kupffer. These granules not only are found near the bordering stellate cells but also appear near the blood sinusoids away from them. Afterward the granules move toward the nucleus, where they become concentrated and then seem to "flow" toward the bile capillaries (Fig. 4). So long as the blood and the stellate cells (according to Radt's experiments, 1930) contain trypan blue, the dye enters the cell over its entire surface, becomes concentrated around the nucleus, and moves along "fixed" pathways toward the bile capillaries, where it becomes extruded. The whole process is a slow one. This is demonstrated by the fact that (*a*) the nuclear accumulation continues to exist for a long period after administration of the dye has been stopped and (*b*) the granular "flow" is much denser near the central accumulation than near the capillaries. Fourteen days after the last (of four) injections, the nuclear accumulation has disappeared, and the "flow" is no longer distinct. However, there still are a few blue granules scattered through the cell. After 4 weeks, practically all trypan blue seems to be eliminated, not only by the liver, however, since the kidneys play the most important part in trypan blue elimination, according to the investigations of von Möllendorff (1915, 1920, 1922).

The cells of the reticulo-endothelial system and the histiocytes which phagocytize trypan blue are relatively short-lived. They degenerate regularly, thus setting the phagocytized dye free again. It is therefore easily understood that even a month after the last injection of trypan blue very small quantities of this dye can still be found in the liver.

The "fixed" pathways along which the dye granules become eliminated suggest the co-operation of cell structures. Nassonov (1926) described a net-shaped osmiophilic structure (Golgi network) in the hepatic cell of the mouse. This structure would connect the various bile capillaries. After intraperitoneal administration of trypan blue, he obtained a "pathway" similar to that described above, which he attributed to a granular adsorption of the dye to the osmiophilic substance. Such pictures of the Golgi substance were, however, never found in my experiments, or by Makarov (1933), Ludford (1928), or Cramer and Ludford (1926/27). Ludford likewise demonstrated in mice individual Golgi fragments between the nucleus and the bile capillaries which would "adsorb" trypan blue. However, according to his drawings, most of the dye granules appear isolated and not adhered to or connected with the Golgi substance. Glasunow (1927) obtained in the guinea pig a distinct blue network that would be the same as the Golgi network. A "pathway" was not mentioned. Neither in man (Pfuhl, 1932) nor in mice (Makarov, Ludford, Sawelsohn, 1929) were such blue networks ever observed. The dye just appears granular,

relatively irregularly distributed, and concentrated in the Golgi "field." Such an accumulation would suggest a certain relation between Golgi substance and dye granules, *but the latter first appear "diffusely" scattered through the cell and only after the administration of a relatively large amount of dye* (3 cc. in 3 days) *do the accumulation and the "flow" become distinctly apparent. The dye granules, therefore, do not appear for the first time at the surface of the Golgi substance or in the "strands."* If a morphological relationship between dye granules and Golgi substance existed, the granular accumulation would move together with the Golgi fragments toward the bile capillaries in certain stages of the 24-hour cycle (5:00–8:00 P.M.). But this is not the case. Even in this period the "flow" always remains much denser near the nucleus. Further, the "flow" would arrive rhythmically, which is not the case. We are therefore forced to conclude that in the hepatic cell of the albino mouse there exists no direct morphological relation between Golgi substance and trypan blue granules, although in certain stages of the 24-hour cycle a topographical relation is apparent.

No relationship between dye granules and mitochondria was ever found. The mitochondria never show any sign of accumulating around the nucleus. The typical nuclear accumulation of dye granules must therefore be the result of the activity of the undifferentiated cytoplasm.

By comparing the mode of elimination of bilirubin (which becomes extruded together with the secretion) with that of semicolloidal substances like trypan blue, a distinct difference is apparent. The bile granules never tend to form nuclear concentrations or to follow definite "pathways." However, bilirubin is an extremely diffusible substance which easily penetrates all kinds of cells (jaundice), whereas trypan blue is not so diffusible. It seems possible that bilirubin leaves the cell together with the "diffuse" secretions and not along a "pathway" of semicolloidal substances. The latter ones seem to be eliminated only after increased cell activity. The hepatic cell of the albino mouse would accordingly possess two different mechanisms of extrusion: first, a "diffuse" system for easily penetrating and diffusible substances and, second, a "pre-formed" system for (semi)colloidal substances which can be eliminated only with difficulty.

SUMMARY

1. There is no indication of any morphological relation between well-known cell structures and bile *secretion*. The Golgi substance becomes reduced during the period of increase of bile formation (11:00 A.M.—8:00 P.M.). In this time the diminishing Golgi fragments move toward the cell surface, especially toward the region of the bile capillaries. Bile seems to originate diffusely all through the cell and very probably is eliminated in submicroscopic amounts. Therefore, the bile granules in fixed tissue represent artifacts.

2. The *excretion* of trypan blue is performed along definite "pathways": first, the dye granules appear scattered through the cell; then they become concentrated around the nucleus, after which they "flow" toward the bile capillaries. Near the capillaries the number of dye granules is definitely smaller than that of the nuclear concentration. Although there seems to occur a topographical relation between Golgi complex and the dye granules in some stages of cell activity, a morphological one could not be

demonstrated. Likewise no relationship could be found between mitochondria, lipochondria, or nucleus and trypan blue (or bile).

3. Two kinds of excretion occur in the hepatic cell of the albino mouse: easily diffusible substances (like bilirubin) are excreted in the same way as the diffuse secretion, whereas (semi)colloidal substances are eliminated along "fixed" pathways.

4. It is obvious that both secretion and excretion are performed by the undifferentiated cytoplasm without any morphologically recognizable interference of the other cell structures.

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